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Silencing Through Heterochromatin Formation: The Role of Piwi in Transposon Targeting

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Silencing Through Heterochromatin Formation: The Role of Piwi in Transposon Targeting

by

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Abstract

Heterochromatin packages a large portion of the genome in eukaryotes, and plays important roles in mitosis, telomere homeostasis, and transposon (TE) control, all necessary to ensure genome stability. A precisely controlled mechanism to designate which regions of the genome should be so packaged is critical to the fitness of an organism. Mis-localized heterochromatin can lead to mis-regulation of nearby and even distant genes, with pleotropic consequences. How cells (and hence organisms) precisely control the location and extent of heterochromatin formation is an intriguing question. Models involving small RNAs, epigenetic inheritance of histone modifications, DNA binding proteins targeting specific motifs, and other mechanisms have been proposed.

Small RNA targeting of heterochromatin formation is an established mechanism in fission yeast (*Schizosaccharomyces pombe*) and the flowering plant, *Arabidopsis thaliana*. However, a parallel mechanism in the animal kingdom has not been clearly established. My thesis work focuses on this issue, using the fruit fly, *Drosophila melanogaster*. Heterochromatin silencing in flies can be monitored using a reporter exhibiting variegating pigmentation in the eyes, i.e. Position Effect Variegation (PEV). The PEV phenotype is known to be quite variable, and I have found that much of the variation has a genetic basis (Chap. 2). Using PEV reporter lines I have investigated a subtype of heterochromatin, the telomeric region of the Y chromosome short arm (Ys). I found that telomeric Ys has a unique response profile to PEV modifiers and appears to employ a distinct type of targeting mechanism (Chap. 3). To further address the question

of targeting, I have engineered knockdown mutations of Piwi, a nuclear-localized small RNA binding protein, and demonstrated a loss of silencing and a corresponding loss of heterochromatin formation at targeted TE's. Genetic manipulations of Piwi and other components of the system confirm that small RNA targeting appears to be one of the mechanisms used in determining the regions subject to heterochromatin formation (Chap. 4). Moreover, Piwi appears to silence transposons through additional mechanisms as well, including playing a cytoplasmic role (Chap. 4, 5).

From my thesis work, I conclude that Piwi has a role in piRNA- based transcriptional silencing of transposons. However, how this mechanism translates to overall heterochromatin formation in the fly genome awaits further investigation. The packaging of the eukaryotic genome appears to be more complex than the picture delineated by the euchromatin/heterochromatin dichotomy. A more complex targeting mechanism, as is suggested by this work, is therefore needed to adequately describe the packaging process.

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Chapter 1

Introduction

Perspective

Heterochromatin formation and maintenance has to be precisely controlled to avoid mutagenic events, such as transposon activation or telomere fusion. It is equally important to avoid ectopic heterochromatin formation, which could potentially perturb developmental programs by silencing unintended genes. I was therefore very intrigued to learn that position effect variegation (PEV) is described as a highly variable phenotype governed by stochastic spreading of heterochromatin, which seems contradictory to the precision required for normal function. Work presented in this thesis looks at PEV, and therefore heterochromatin silencing, from a deterministic perspective, aiming to identify what is determined in heterochromatin silencing and what the determining factors are. The key question being how heterochromatin is properly localized to execute its function, given its stochastic nature.

This thesis is composed of six chapters representing three different ways of approaching this question. In the second chapter I look at the correlation between the variations of the PEV phenotype with the variations in the genome. It is to some extent surprising and to some extent reassuring to find a high degree of correlation between the two. Most of the variation in the PEV phenotype is determined by the genome (genetic background). The question remaining is what are the relevant determining factors in the genome. In the third chapter I look at some of the potential genetic factors impacting the PEV

phenotype of a particular domain in the genome, the Ys telomere. The results indicate yet another subtype of heterochromatin. It appears that while much of a PEV phenotype is determined by genetics, a simple model capable of describing all that we know about heterochromatin is intangible. Despite the pessimism, in chapters four and five I look at the same question from a reductionist's point of view. I focus on a potential targeting mechanism for heterochromatin formation to better understand how heterochromatin is localized in the genome. Work on Piwi has revealed a potential mechanism of small RNA targeting of transposon sequences in heterochromatin formation.

The main body of this first chapter is composed of a review focusing on the current progress toward understanding heterochromatin targeting mechanisms in flies, with a particular emphasis on small RNA targeting of transposon sequences. The first half of the review discusses the cis-acting elements potentially responsible for seeding heterochromatin formation (the targets). The second half of the review focuses on potential trans-targeting machineries, which puts the results presented in chapter four and some of chapter five into the context of current literature. Although this review does not directly mention the results from chapters two and three of the thesis, all of the discussion is based on a genetically determined view of heterochromatin silencing. Much of the discussion leads to a complex picture of heterochromatin silencing, which resonates well with the observations made in chapter three.

Contributions to the manuscript

This manuscript has been prepared as an invited review for the journal Biochemistry (Moscow). As a co-first author, I wrote the first draft of the section on trans-acting machineries and most of the concluding remarks. Monica Sentmanat wrote the first draft of the section on cis-acting elements, and most of the introduction. I was also involved in revising the entire manuscript together with the other co-authors (MS and SCRE).

Targeting heterochromatin formation to transposable elements in Drosophila: potential roles of the piRNA system

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ABSTRACT

Heterochromatin formation is critical for genome stability in eukaryotes. Properly localized heterochromatin is required for the normal progression of development. Here we focus on heterochromatin assembly mechanisms in *Drosophila melanogaster*. In particular, we review the potential role of transposable elements as genetic determinants of the chromatin state, and examine how small RNA pathways may participate in the process of targeted heterochromatin formation.

INTRODUCTION

Cytological staining of interphase chromatin reveals two apparent states of compaction – a euchromatic state which is lightly stained, diffuse in appearance; and a heterochromatic state which is densely stained, appearing compact (Zacharias, 1995). Thus heterochromatin is classically defined as densely packaged throughout cell cycle, peripherally localized nuclear material. The repetitious sequence content of eukaryotic genomes was initially recognized by quantitative DNA reassociation analysis (or Cot curves) using principles pioneered by Roy Britten and colleagues (Britten and Kohne, 1968). These studies revealed the abundance and arrangement of repetitive DNA, and ultimately led to the understanding that heterochromatin is enriched in satellite and transposable element sequences of varying copy numbers. Although understanding genome organization within the euchromatic, more complex, gene-rich compartment took precedence for many years, heterochromatin has more recently received attention with the development of improved sequencing

technologies and bioinformatics strategies. These tools have enabled improved assemblies and annotation of repeats present in heterochromatin.

In a complex organism consisting of differentiated cells, “constitutve heterochromatin“ is that found at the same place in all cell types, while “facultative heterochromatin“ (important for developmentally controlled genes) occurs in some cells but not others. Along a chromosome, constitutive heterochromatin is usually found at pericentric repeats and telomeres, while facultative heterochromatin can be interspersed along the chromosome arms. Heterochromatin is generally characterized by a signature of histone modifications that includes H3K9me2/3; in plants, mammals, and some other organisms it is also associated with DNA methylation at CpG or CpNpG repeats.

In the fruit fly, *Drosophila melanogaster*, heterochromatin becomes visible during nuclear cycle 11-14 of embryogenesis (3-4 hrs), establishing post-translational histone modifications that persist throughout development (Hathaway et al.). Most heterochromatic sites are enriched for H3K9me2/3, the chromo domain protein HP1a and the histone methyltransferase (HMT) SU(VAR)3-9, whose catalytic SET domain delivers the H3K9me2/3 mark. Two other SET domain proteins have been identified, SETDB1 (encoded by *egg*) and G9a; both are also H3K9 histone methyltransferases, although Su(var)3-9 and Egg appear to have the dominant role (Brower-Toland et al., 2009).

Functional studies that deplete SU(VAR)3-9 homologues in mammals or in yeast have shown that the protein is important for kinetochore assembly and

chromosome segregation (Aagaard et al., 1999; Ekwall et al., 1996), while a loss of HP1a in Drosophila results in telomere fusions (Fanti et al., 1998). Another form of instability from the loss of heterochromatin (HP1a in particular) is the activation of transposable elements (Wang and Elgin, 2011), which leads to double strand breaks as well as the obvious mutagenizing effects of TE insertions within protein-coding DNA. Gain-of-function mutations in *Su(var)3-9* cause heterochromatin expansion and female sterility in Drosophila (Kuhfittig et al., 2001). Alternatively, facultative heterochromatin proteins play an important role in cell identity. Examples include X-inactivation in mammals and developmentally controlled silencing progams associated with Polycomb group (PcG) proteins, which accomplish targeted gene silencing using an H3K27me3-based mechanism. This review will primarily focus on mechansims associated with HP1a targeting. Our discussion of “heterochromatin“ will be in reference to constitutive hetrochromatin unless otherwise specified. In Drosophila, the constitutive heterochromatin domains include the pericentric heterochromatin, regions in the telomeres, and the bulk of the small fourth chromosome (Muller F element) (Kharchenko et al., 2011).

A classic and commonly used assay to dissect the cis- and trans-acting factors involved in heterochromatic silencing in Drosophila (among other systems) involves position-effect variegation (PEV) – first observed in Drosophila by Herman Muller in the 1930s. Following X-ray mutagenesis, Muller recovered fly lines (termed w^{m}, *white mottled*) that had a variegating, red-interspersed-with-white pattern across the fly eye, rather than its normally solid-red (or completely

white, if mutant) appearance (Muller, 1930). The phenotype is caused by a DNA rearrangement that places the euchromatic *white* gene, which has a transport function required cell-autonomously for red eye pigmentation, proximal to repeat-rich pericentric heterochromatin. This results in the stochastic “spreading” of heterochromatin components along the now proximally located euchromatic mass that includes *white* (**Fig. 1A**). Dominant loss-of-function mutations in heterochromatin components such as *Su(var)3-9* or the HP1a gene *Su(var)205* suppress the PEV phenotype such that the expression of *white* is restored in a greater fraction of cells, whereas over-expression can have the opposite effect. At the chromatin level, PEV is characterized as resulting in a relatively regular nucleosome array (Sun et al., 2001; Wallrath and Elgin, 1995), indicative of heterochromatic packaging. Biochemical analysis across the inverted breakpoint of one strain from the w^m collection, w^{m4}, shows variable enrichment of heterochromatin proteins along a 30 kb stretch, suggesting some sequence determinants might be more susceptible than others to ectopic heterochromatin assembly (Vogel et al., 2009). Together, these observations suggest that heterochromatin assembly can spread in *cis*- provided a permissible sequence context and sufficient trans-acting molecules. These properties have made PEV a widely used model with which to dissect the cis- and trans-acting factors responsible for heterochromatin assembly.

Localized distribution of heterochromatin in the genome implies an underlying sequence determinant for its targeted formation. The immediate question following this observation asks for a mechanistic explanation for the

targeting process. In recent years, work from plants and the fission yeast *S. pombe* have established that many of the heterochromatin components in these systems are associated with RNA-directed transcriptional silencing (Slotkin and Martienssen, 2007). In these systems, RNA transcribed from repetitive, heterochromatic loci is processed into small RNAs that ultimately become the targeting signal for heterochromatin assembly. Such a targeting mechanism, in which the targeting signal is generated from heterochromatin (the target) itself, allows plasticity. This is necessary to accommodate imprecision during DNA replication or new TE invasions that change the system's DNA composition, while ensuring functional precision (faithful heterochromatin assembly).

S. pombe, a system for which RNA-directed transcriptional silencing is well described, serves as an excellent model of how *cis*-sequence determinants work with *trans*-acting factors to assemble heterochromatin at repeats, generally remnants of transposable elements (TEs). Targeting of the HP1 family protein Swi6 and the H3K9 HMT Clr4 depends on the processing of RNA Pol II transcripts generated from heterochromatic loci. The RNAi-induced transcriptional silencing complex (RITS) contains the chromo domain protein Chp1, as well as the RNAi component Ago1, which binds small RNAs generated from target sites (e.g. *dg/dh* repeats, *cis*-acting signals) located in pericentric heterochromatin (Kloc and Martienssen, 2008) (**Fig. 2**). Mutations in the slicer activity of Ago1 result in a loss of silencing for reporters located at heterochromatic sites (Irvine et al., 2006), indicating that Ago1 is an essential trans-acting factor for heterochromatin assembly in *S. pombe,* and that

processing the long RNA cis-acting signal from *dg/dh* repeats into smaller fragments is required. The small RNAs generated by Ago1 provide a primer for RNA-dependent RNA polymerase, which generates additional dsRNA products to be processed by Dicer1. The amplified small RNA is used to achieve additional RITS targeting. However, whether such a mechanism also operates in metazoan systems remains an open question.

It is important to distinguish between RNA-based silencing systems (here referred to as RNA interference or RNAi), which are associated with post-transcriptional mRNA silencing, and those implicated in chromatin-based silencing (**Fig. 3**). In *Drosophila*, RNAi primarily involves two families of proteins: Argonaute proteins, AGO1, AGO2, AGO3, Piwi and Aub, and RNase III helicases, DICER-1 and DICER-2. The Argonaute family comprises two clades, the more ubiquitous AGO clade (AGO1 and AGO2) and the primarily germ line PIWI clade (AGO3, Aub and Piwi). AGO1 and DICER-1 generate microRNAs, derived from imperfect stem-loop transcripts, that participate in translational repression or degradation of mRNA target transcripts. Short-interfering RNA (siRNA) is derived from exogenous or endogenous (endoRNAs) dsRNA processed by AGO2 and DICER-2 (Ghildiyal et al., 2008; Kawamura et al., 2008). Although siRNA is generally considered to function through a post-transcriptional silencing mechanism in the cytoplasm, both AGO2 and DICER-2 have recently been documented to associate with chromatin in somatic nuclei, suggesting a role in nuclear silencing (**Fig. 3**) (Cernilogar et al., 2011; Moshkovich et al., 2011). PIWI-interacting RNAs, piRNAs, are derived from

master clusters enriched in transposon sequences (Brennecke et al., 2007; Gunawardane et al., 2007). Both transcriptional and post-transcriptional silencing mechanisms have been reported for transposon silencing by piRNA (**Fig. 3**) (Brennecke et al., 2007; Gunawardane et al., 2007; Klenov et al., 2011; Wang and Elgin, 2011). .

In spite of their hazardous potential, transposons are among the genome's most important tools, providing the host new material for cis-acting regulatory features and protein-coding capacity (Feschotte, 2008). The paradox between a necessity to maintain genome integrity, while also achieving diversity within a population has been empirically linked to RNAi-mediated transposon regulation (Gangaraju et al., 2010). Indeed, such mechanisms have been speculated to participate in generating new variants in a changing environment, with profound consequences over the evolutionary trajectory of the population. Thus, RNAi systems in Drosophila, particularly the piRNA pathway, can be thought of as a master regulatory switchboard, with the primary task of TE repression. Whether these effects occur at the chromatin level is the topic of this review.

We aim to synthesize the evidence for RNAi-induced heterochromatin targeting in Drosophila. In particular, we focus on repetitious elements acting as cis-acting signals. We begin by discussing established examples of cis-acting silencing signals, which serve as precedents for sequence-specific targeting of chromatin modifying enzymes. Although many empirical examples exist that involve transcriptional activation (Feschotte, 2008), we explore the potential of

TE remnants to act as silencing signals to be used by RNAi pathway component effectors.

CIS-ACTING ELEMENTS

Cis-acting factors that are targets for complexes that bind, rearrange, and/or modify histones have profound effects on nucleosome organization and higher-order interactions. A classic example are polycomb response elements (PREs), cis-acting DNA sequence targets. PREs are targets for the developmentally controlled Polycomb group (PcG) repressor complexes PRC1, PRC2, and PhoRC (Muller and Kassis, 2006), responsible for one form of facultative silencing. Although these complexes contain histone binding and modifying subunits, it is the cis-acting sequence content present in PREs that is required for appropriate targeting. Indeed, PREs have been found to be nucleosome-free assembly platforms (Muller and Kassis, 2006), supporting a sequence-specific targeting event (as opposed to a modified histone-protein interaction). Reporter assays using upstream putative regulatory regions of the animal polarity-determining Hox genes identified PREs as necessary sequence components for targeted gene silencing (Chan et al., 1994; Simon et al., 1993). In flies, genome-wide analysis of the sequence composition of PREs has revealed low conservation (Hauenschild et al., 2008), with individual PREs possessing inherently different propensities for silencing (Okulski et al.). The low sequence conservations has been suggested to impart a certain degree of

plasticity to these sites which enables them to evolve rapidly (Moazed, 2009).

TEs are an abundant resource for potential cis-regulatory elements. Transposable elements have the ability to retool their host's gene regulatory programs, and so to contribute to networks involved in cell identity during tissue specialization, much like PREs. The capacity of TEs to establish novel gene regulatory networks, particularly species-specific programs that contribute to new evolutionary trajectories, is supported by much empirical evidence (Feschotte, 2008). Although such new networks are fortuitous for the system, particularly under times of environmental stress, it is generally in the best interest of genome integrity for TE expression and mobilization to remain suppressed. Transposable elements and their remnants comprise 22% of the Drosophila genome (Kapitonov and Jurka, 2003) and roughly half of the human genome (Lander et al., 2001); they reside primarily in repressive, heterochromatic regions. The non-random distribution and evolutionary conservation of heterochromatic TE clusters suggests that their residence is functionally required. As previously discussed, TEs inherently possess regulatory signals or may acquire them *de novo*; this, combined with their capacity for insertional mutagenesis, more often than not results in a substantial blow to the system during mobilization events. Thus, repression of these elements takes precedence under most circumstances. Indeed, the flux of TEs in the genome requires a rapidly adaptive targeted silencing system for survival. Deep sequencing of small RNA libraries has shown that TEs are expressed, and become targets for small RNA-mediated silencing in flies (Brennecke et al., 2007; Ghildiyal et al., 2008). Although small RNA

pathways are better known for their function in a post-transcriptional capacity, evidence for chromatin-based silencing in Drosophila has been reported (Huisinga and Elgin, 2009). Both piRNA and chromatin structural proteins (and/or their mRNAs) are present in the early embryo (0-6 hr) (Aravin et al., 2003) during the early stages of heterochromatin formation (Rudolph et al., 2007). Thus, piRNA sequence elements could help define some heterochromatic domains, particularly for a subset of repeats represented in the piRNA repretoire.

Chromosome organization *per se* suggests that TEs could be targets for silencing, as many Drosophila PEV reporters showing the variegating phenotype typical of heterochromatic domains map to repeat-rich regions of the genome. Studies aimed at mapping heterochromatic domains on the repeat-rich 4th chromosome of *Drosophila melanogaster* using an *hsp70-white* reporter have shown that 20-60 kb deletions or duplications of flanking DNA can be sufficient to shift a red phenotype to vareigating (and vice versa), indicating local variation in chromatin packaging at that scale (Sun et al., 2004) (**Fig. 1B**). Genomic analysis of these variegating lines found a correlation between the presence of the DNA transposable element *1360* and silencing. Follow-up experiments using FLP-mediated excision of a *1360* remnant upstream of an *hsp70-white* reporter revealed that *1360* is indeed capable of supporting heterochromatin formation predominantly in repeat-rich areas of the genome (~30% repeats) (Haynes et al., 2006). Interestingly, *1360* is sufficient to induce ectopic, HP1a-dependent heterochromatin assembly in a domain of annotated euchromatin that is close to a heterochromatic mass (Sentmanat and Elgin, 2012). Variegation in both

contexts, repeat-rich and euchromatic, is suppressed in *Su(var)205* and *piwi* mutants, suggesting that RNAi components may facilitate the HP1a targeting event. RNAi-based heterochromatin targeting in both *S. pombe* and plants is thought to act through RNA-RNA recognition events. A mechanistic connection between such transcriptional silencing and *1360*-induced heterochromatin assembly was observed when read-through transcripts of the P element insert containing *1360* were found to be present in 0-10 hr embryos, suggesting a plausible RNA targeting signal. Further, deletion of sites within the *1360* element with homology to piRNA sequences abundantly found in Drosophila compromised *1360*-induced PEV. These results directly implicate the piRNA pathway in *1360*-induced silencing (Sentmanat and Elgin, 2012).

Given that the piRNA pathway generates the most complex small RNA population in the fly – needed to target hundreds of TEs - it is likely that alternative TEs should behave similarly at a *1360*-sensitive site. This was confirmed using the retroelement *Invader4*, which recapitulated *1360*-sensitive PEV. Deletion of sites complementary to piRNA sequence elements again compromised the effect (Sentmanat and Elgin, 2012). The combined results support a model in which a small RNA targeting event utilizing read-through transcripts participates in the HP1a-dependent assembly of heterochromatin at this site.

Sites sensitive to *1360* appear to be limited to sites proximal to pericentric repeats, or in some cases within mapped pericentric regions. As noted above, the presence of a single copy of *1360* within the euchromatic arms (which have a

low repeat density, <10%) is insufficient to trigger a variegating phenotype. A survey of *1360*-sensitive and –independent (no change in PEV +/- *1360*) lines revealed that PEV reporters close to the base of the 2L euchromatic arm are consistently suppressed by *piwi* mutations and almost all are *1360*-sensitive. Many PEV reporters that show no change in variegation in *piwi* mutant backgrounds are *1360*-insensitive and reside in regions associated with polycomb group proteins (TAS sequences). These observations suggest that piRNA pathway target sites are likely HP1a-target sites (as *1360*-sensitive silencing is an HP1a-dependent phenomenon), but limited to a subset of domains. The need for a reporter insertion site that results in read-through transcription of the *1360* element could also limit the set of reporter loci demonstrating this form of targeted silencing.

The repertoire of possible cis targets for the piRNA system is wide, but few elements have been associated with chromatin-based changes in *piwi* mutants. Knockdown of germline Piwi has been shown to compromise HP1a deposition at promoters of HeT-A, Blood, Bari1 and Invader1, among a small set of TEs tested in Drosophila ovaries (Klenov et al., 2011; Wang and Elgin, 2011) (Chapter 4). The lack of sufficient polymorphisms among repetitive element types makes it difficult to identify the precise location of HP1a loss. Thus, the high copy number and lack of complete genome assembly in heterochromatic regions has hampered efforts to identify additional targets. Genomic context at a larger scale (at least over 10 kb, and perhaps much more) may prove to be an important

factor in identifying additional *cis*-acting determinants of heterochromatin formation.

TRANS-ACTING MACHINERIES: SMALL RNA TARGETING

A small RNA-mediated targeting model (Huisinga and Elgin, 2009), representing a mechanism of remarkable simplicity and adaptability, uses sequence information encoded in small RNAs to achieve highly specific target site recognition. The coding capacity of a 20-30 nucleotide long RNA allows a wide range of potential target sequences to be identified. Recently, both endo-siRNA and piRNA have been implicated in heterochromatin targeting (Fagegaltier et al., 2009; Wang and Elgin, 2011). In both cases, however, many critical questions remain to be clarified, in particular, whether changes observed at the chromatin level in endo-siRNA and piRNA pathway mutants are a result of direct or indirect effects. The potential redundancy and/or cross talk between the two pathways further confounds our ability to interpret results from genetic perturbation experiments.

In flies, endo-siRNAs were first observed by sequencing small RNAs associated with AGO2 and small RNAs bearing 2'O-methylation at their 3' terminus from somatic cells (Ghildiyal et al., 2008; Kawamura et al., 2008). It was found that these small RNAs are enriched in transposon and intergenic sequences, and that their production is strongly impacted by mutations disrupting the siRNA pathway. Interestingly, the involvement of these small RNAs in heterochromatin targeting had been implicated even before their identification. It

had already been shown, mostly by cytological assays, that mutations in *ago2* result in defects in centromeric heterochromatin formation (Deshpande et al., 2005). Given the well-established role of AGO2 in a small RNA-based silencing mechanism, and a potential parallel mechanism in *S. pombe* (describing small RNA targeting of heterochromatin formation), these observations pointed to the enticing possibility of siRNA targeting for heterochromatin formation. The model is particularly attractive when taken together with the observed enrichment of transposon sequences in endo-siRNAs.

A test of this model, looking at perturbation of heterochromatin formation and targeting under conditions where endo-siRNA production is disrupted, provides encouraging support. It has been shown that both viral protein sequestering of endo-siRNA, and mutations impacting endo-siRNA production, have a dominant suppression effect on a stubble PEV reporter, Sb^{V} (a translocation of *Sb* to the 2R pericentric region) (Fagegaltier et al., 2009). It has also been shown that trans-heterozygous mutations in components needed for endo-siRNA production, such as AGO2 and DCR2, also show strong suppression of w^{m4} PEV. In addition, in the same study Fagegaltier and colleagues further demonstrated that endo-siRNA component mutations have an impact on localization of HP1a and H3K9me2/3 using immuno-fluorescent staining of polytene chromosomes. While for a good percentage of samples examined, a clear impact on heterochromatin distribution is observed, it should be noted that pericentric heterochromatin remains visibly stained in all cases. These results argue that while the endo-siRNA pathway is critical in determining

the localization pattern of heterochromatin, the specific targeting of heterochromatin formation at the pericentric region is either independent of the endo-siRNA pathway or (more likely) the role of endo-siRNA in this process is redundant with other mechanisms. It is interesting to note that while dominant mutations of these same genes have little to no impact on PEV at some reporter sites (Haynes et al., 2006), inserts of reporter transgenes in other regions of the genome show significant suppression (Wang et al., 2012) (Chapter 3). It appears that involvement of endo-siRNA in targeting heterochromatin formation could be context dependent.

One conundrum of the endo-siRNA targeting model for heterochromatin formation is the fact that siRNA pathway is better known for its function in post-transcriptional silencing in the cytoplasm. It is therefore difficult to draw a direct mechanistic link to a nuclear targeting process for heterochromatin. However, two recent studies have independently demonstrated chromatin-bound AGO2 protein (Cernilogar et al.; Moshkovich et al.), albeit in larval or adult tissues. Although a direct mechanistic link is still missing (i.e. it remains unclear what is recruited by AGO2 to initiate heterochromatinization), endo-siRNA pathway is clearly involved in the process of heterochromatin formation, at least in certain regions of the heterochromatic genome.

Amongst the five Argonaute proteins in the fly genome, the one conspicuously localized in the nucleus is Piwi, of the PIWI family proteins (Brennecke et al., 2007). Piwi has therefore been regarded as the primary candidate Argonaute protein for heterochromatin targeting in Drosophila. The

PIWI proteins associate with piRNAs, 26-30 nt small RNAs that are enriched for TE sequences. Piwi and Aub primarily bind antisense piRNAs derived from "piRNA loci", postulated to be discrete regulatory loci that can be several kilobases long, proposed to generate a transposon defense system. In 2007, two groups independently proposed that a 'ping-pong' amplification loop is responsible for piRNA biogenesis (Brennecke et al., 2007; Gunawardane et al., 2007). piRNA master regulatory loci and endo-siRNA clusters predominantly map to the edges of pericentric and telomeric regions—which are highly enriched in repeats and transposable elements. Work from Pal-Bhadra and colleagues have demonstrated that mutations in PIWI family proteins impacts two types of PEV at multiple genomic loci (Pal-Bhadra et al., 2004). In a study of *Spn-E*, a putative helicase involved in the piRNA pathway (**Fig. 3**), Gvozdev and colleagues demonstrated an impact on heterochromatic structure at transposon sites due to this perturbation of the piRNA pathway (Klenov et al., 2007).

Further evidence supporting the piRNA-targeting model comes from biochemical experiments showing a direct interaction between Piwi and HP1a (Brower-Toland et al., 2007). Additionally, it has been demonstrated that the direct interaction between Piwi and HP1a is dependent on the PXVXL motif at the Piwi N-terminus. A point mutation in this domain disrupts the interaction between Piwi and HP1a in a yeast two-hybrid setting and *in vitro* (Mendez et al., 2011). This observation connects the targeting model directly to the well-established HP1a-centric model for the spread of heterochromatin (Girton and Johansen, 2008), and provides a theoretical framework for understanding the

heterochromatin formation process in flies.

Piwi was first described to be involved in the maintenance of germline stem cells (Cox et al., 1998). This function was shown to be required in the stem cell niche of ovarian soma. Deep sequencing of piRNA initially positioned Piwi alongside Aub in the Ping-Pong amplification cycle for generating secondary piRNAs (Brennecke et al., 2007). This model was later modified in response to results from sequencing piRNA in *piwi* mutant ovaries (Li et al., 2009), and the role of Piwi in generating piRNAs became obscure. A functional test of the piRNA targeting model for heterochromatin formation in the female germline demonstrated a function for Piwi downstream of piRNA production in deposition of HP1a at the putative promoter region for most of the transposons tested (Wang and Elgin, 2011) (Chapter 4). This interpretation is supported by an independent study using an N-terminal truncation mutant of Piwi, which fails to localize in the nucleus, to demonstrate the critical function of Piwi nuclear localization in transposon silencing and enrichment of heterochromatic markers at a subset of transposon sites (Klenov et al., 2011). Taken together, results from these two studies and a previous observation from Saito and colleagues, on the dependency of Piwi nuclear localization on piRNA binding (Saito et al., 2009), make a compelling case that piRNA targeting of Piwi plays a role in transcriptional silencing of transposons.

Evidence supporting the transcriptional silencing model for Piwi-dependent transposon suppression also arises from an independent report showing an increase in HeT-A transcription using nuclear run-on assays

performed in ovaries depleted for Piwi (Shpiz et al.). It should be noted that an earlier report from Zamore and colleagues found a lack of impact on the transcription rate of transposons (e.g. *mst40*) in *armitage* mutants, suggesting a post-transcriptional silencing mechanism for piRNA in transposon silencing (Sigova et al., 2006) (Vagin et al., 2006). Consistent with this observation, silencing of the transposon Jockey is not impacted by HP1a depletion, indicating that it is not regulated by a chromatin-based mechanism, even though it is dependent on Piwi (Wang and Elgin, 2011) (Chapter 4). Thus, a post-transcriptional component is clearly part of the piRNA silencing mechanism, and maybe particularly relevant to a subset of TEs. However, given the predominant nuclear localization pattern of Piwi, and the concordance between TE over-expression and depletion of HP1a at these TEs, we argue that a transcriptional silencing mechanism mediated through a piRNA-directed heterochromatin targeting process is a major mechanism for transposon silencing by piRNA.

The physical interaction between Piwi and HP1a that connects the targeting model with the spreading model of heterochromatin formation is a substantive link. However, an attempt to verify the importance of this direct interaction in transposon silencing *in vivo* led to the discovery of unexpected complexities. By substituting the wild type Piwi in the germline with a single residue mutant form (V30A) which fails to interact with HP1a in a yeast two-hybrid experiment had no obvious impact on transposon silencing (Wang and Elgin, 2011) (Chapter 4). It was hypothesized that additional proteins bridge the Piwi and HP1a interaction, perhaps in a way similar to Tas3 in the *Pombe* RITS

complex, and that this creates a more robust system. Further biochemical work will likely be needed to yield insights into these interactions. Alternatively, other chromosomal proteins than HP1a might be initially targeted to the TEs. A tudor-domain containing histone methyl-transferase, EGG, appears to be a promising alternative candidate for Piwi targeting of heterochromatin formation; this key protein is prominently associated with piRNA loci, and necessary to maintain their heterochromatic status (Rangan et al., 2011).

In future studies, experiments using constructs bypassing the need for small RNA targeting of Piwi to induce heterochromatin formation could be informative in deciphering how Piwi recruits relevant downstream factors, if indeed it does. Ectopic tethering of a wild type or PAZ domain mutant form of Piwi is being tested in an attempt to induce ectopic heterochromatin formation. A strong claim could potentially be made from this type of sufficiency, but the results from these experiments may be difficult to interpret due to the context-dependent nature of heterochromatin silencing. Given the discussion above, a context-dependent impact of tethering is the likely outcome.

One critical question concerning the piRNA targeting model for heterochromatin formation stems from the fact that piRNA is thought to be restricted to the reproductive system and the early zygote (Brennecke et al., 2007). However, heterochromatin is critical for maintaining genome stability and adequate chromosome segregation during mitosis throughout the lifetime of the individual; thus, the lack of a heterochromatin targeting/assembly mechanism in most tissue types does not seem plausible. While the endo-siRNA pathway could

potentially be an alternative targeting mechanism in the soma, many of the studies cited above show an impact of piRNA component mutations in the larval and adult tissues normally scored in PEV assays. It remains unclear how mutations in genes that are not known to be expressed could impact chromatin structure in those tissues. One intriguing possibility is the epigenetic inheritance of chromatin structure through mitosis. Heterochromatin formation is first observed during embryonic stage four (nuclear cycle 11-14) and is thought to maintain complete silencing until the relaxation phase during the late third instar larval stage (Lu et al., 1998). It is conceivable that the impact of Piwi depletion in the early zygote could be maintained epigenetically through mitosis and lead to the observed phenotype in later developmental stages of the zygote. In fact, a recent study has shown such an impact upon the conditional depletion of Piwi in the early zygote by RNAi knockdown; a strong impact on suppression of PEV is visible in adults (Tingting Gu and SCR Elgin, personal communication). While significant depletion of HP1a is apparent at the reporter site, the impact on heterochromatin as a whole appears to be minimal, suggesting how these animals might display a visible phenotype while maintaining the minimum required heterochromatin for the progression of the developmental program. We note that while the TEs are an important component of heterochromatin, satellite DNA sequences are also a significant part of the whole, and might be targeted by other mechanisms. Studies of mitotic inheritance upon ectopic heterochromatin formation induced by conditional (temporal) tethering of Piwi could provide a strong argument for the epigenetic inheritance model described here.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Here, we focused our discussion on the targeting aspect of heterochromatin formation. We reviewed the tremendous progress in the past decade on this issue using the fruit fly as a model organism. Clearly, small RNAs are instrumental in the targeting process required to silence transposons. However, a reoccurring theme throughout the review is that most of the reported experimental observations are dependent on genome context (proximity to heterochromatic masses, etc), thus making the derivation of a general rule difficult. For example, the impacts of mutations in the genes for RNAi pathway components show a differential response when tested on PEV reporter inserts present in different genomic loci. This no doubt reflects the mosaic nature of heterochromatin, and could also relate to the special features of the piRNA loci, which are certainly packaged as heterochromatin in somatic cells (Kharchenko et al 2011). The effectiveness of *1360* to enhance or drive HP1a-dependent silencing also varies depending on the site tested (see discussion above). It is apparent to us that complex interactions between multiple mechanisms must be in place, preventing us from deriving simple rules from our observations. From an evolutionary point of view, the involvement of transposons in this process almost guarantees a convoluted mechanism like the one we observed. There is no doubt an ”arms race” between the host species and the invading transposable elements through the evolutionary time scale, similar to that reported for viral defense systems. Whichever strategy succeeds in helping the host cope with the

invasive new transposon will result in a further (potentially redundant) mechanism built into the system.

The idea of heterochromatin targeting originated from a vision in which only two types of chromatin exist in the genome. In this scenario, while the majority of the genome is composed of euchromatin, the formation of the densely localized heterochromatic regions must be specifically targeted. The dichotomous classification of chromatin structure, while a good starting point and still useful in many cases, is insufficient to describe observations made from recent experiments. Domains and subtypes of heterochromatin have therefore been reported to describe the differences between pericentric and telomeric heterochromatin (Cryderman et al., 1999; Doheny et al., 2008). More recently, results from genome-wide chromatin immunoprecipitation mapping of chromosomal proteins and histone modifications has suggested other informative ways of classifying chromatin structure across the genome. For example the nine-state model can be used to adequately identify enhancer regions, transcription start sites and polycomb-regulated regions in addition to classic heterochromatin (Kharchenko et al., 2011). These new additions to our knowledge have in many ways made the euchromatin/heterochromatin dichotomy obsolete. Consequently, the heterochromatin targeting model built from this vision must be revised to accommodate a more nuanced perspective.

A multiplicity of targeting mechanisms for sites with similar chromatin marks has been observed in systems that possess well-documented RNAi-mediated transcriptional silencing, such as *S. pombe* and *N. crassa*. This should

come as no surprise; in light of the complex chromatin environments present in a genome (Kharchenko et al., 2011), equally complex targeting systems have been developed. In *S. pombe*, all of the major heterochromatic domains are targeted for silencing by proteins recognizing specific DNA sequences, in addition to the RNAi-based mechanisms (Slotkin and Martienssen, 2007). Conversely, TE elements have been used for different recognition events as well. Transposase-derived chromatin modifers have been documented in Drosophila as well as in mammals. For example, BEAF-32, derived from the *hAT* transposase, is a chromatin insulator protein that binds the *scs* chromatin boundary element (Aravind, 2000). Similar mechanisms may have evolved in Drosophila to specifically target heterochromatin factors to TEs. One possible candidate (but with no known transposase-derived domains) is Bonus, a Tif1 homolog that derives from a family of proteins identified to interact with HP1 and recruit HDACs to mediate transcriptional repression (Nielsen et al., 1999). In Drosophila, Bonus can suppress or enhance PEV, depending on the reporter insert, and binds repetitive sequence elements in euchromatin (Beckstead et al., 2005). This suggests that Bonus has a role in chromatin organization, but precisely what that is remains an open question. In mouse ES cells, the Bonus homolog Kap1 is required to maintain H3K9me3 at endogenous retroviral elements, a consequence of a sequence-specific binding event, as the 5'UTR of an ErV element is sufficient to induce KAP1-dependent silencing of a GFP reporter.

The AT-hook, DNA binding protein D1 has been found to localize to centromeric heterochromatin and suppress PEV (Aulner et al., 2002). Genome-

wide mapping analysis has revealed that D1 overlaps with several combinatorial categories of chromatin marks that can be generally ascribed to silent chromatin, in particular, HP1a-dependent heterochromatin and PcG-associated silencing (Filion et al., 2010). However, as is often the case, layers of complexity emerge as others report no dominant suppression of PEV by *D1* (Weiler and Chatterjee, 2009).

While there is no doubt that certain targeting events are needed to ensure proper heterochromatin silencing, as supported by ample evidence reviewed in this introduction, the pursuit of a single unifying mechanism in heterochromatin targeting is likely to be futile. We propose, instead, that multiple mechanisms function in a complex network to ensure proper chromatin structure formation in the genome. This complex interactive network forms the basis of the context-dependent effects that we so often see in genetic dissections of chromatin biology. Towards a better understanding of chromatin based gene regulation, perhaps the reductionist approach, seeking simple explanations for targeting mechanisms should be replaced. To gain predictive power on the outcomes from simple perturbation experiments, we will have to embrace the inherent complexity of the system and utilize the wealth of genomic information derived from high throughput technologies. Where possible, this philosophy has been applied in the studies that follow.

FIGURE LEGEND

1.Position-effect variegation in *Drosophila melanogaster.* Schematic depiction of

the chromosomal inversion generating the white-mottled four line (*In(1)w^{m4}*) by Muller (Muller, 1930), that places the euchromatic *white* gene (coding for a transporter protein required for red eye pigment) adjacent to pericentric heterochromatin. The light red bar represents heterochromatin while the light green bar represents a euchromatic chromatin state. The chromosomal inversion results in silencing for some cells (white, due to heterchromatin spreading over the *w* gene) and expression in others (red).

2. RNAi-transcriptional silencing in *S. pombe*. Transcripts of *dg/dh* pericentric repeats are targeted by the RNA-induced transcriptional silencing complex (RITS). RITS consists of the chromo domain protein Chp1, Tas3 and the small RNA associated protein Ago1. A second complex, the RNA-directed RNA polymerase complex (RDRC) consists of the RNA-directed RNA polymerase 1 (Rdp1), a putative polyA polymerase Cid12 and helicase Hrr1. RDRC is recruited to *dh/dg* repeats by a physical interaction with RITS to synthesize double stranded RNA, which are targeted by Dicer to make additional siRNAs to reinforce RITS recruitment.

3. Small RNA-mediated silencing in *D. melanogaster*. Only siRNA and piRNA pathways are illustrated. Note that while the piRNA pathway is more restricted to the reproductive system, the siRNA pathway has a broader distribution. Both pathways have been implicated in a small RNA mediated heterochromatin targeting process. In the siRNA pathway, small RNA generated by Dcr2 is loaded to AGO2 RISC. The AGO2 complex can suppress expression via either slicing target mRNA in the cytoplasm through a well-characterized post-transcriptional

gene silencing (PTGS) mechanism or through a yet to be characterized chromatin-based transcriptional silencing mechanism (TGS) in the nucleus. In the piRNA pathway, primary piRNA generated by a process involving Zuc is fed into the Ping-Pong cycle involving Aub and AGO3 to generate secondary piRNA. This step is proposed to function simultaneously in amplifying antisense secondary piRNA and suppress transposon expression via slicing. *Spn-E* is required for secondary piRNA production although the detailed mechanism is unclear. Secondary piRNAs loaded onto Piwi, likely by Armitage, allows nuclear localization of Piwi and downstream recruitment of HP1a to induce heterochromatin silencing of transpososns.

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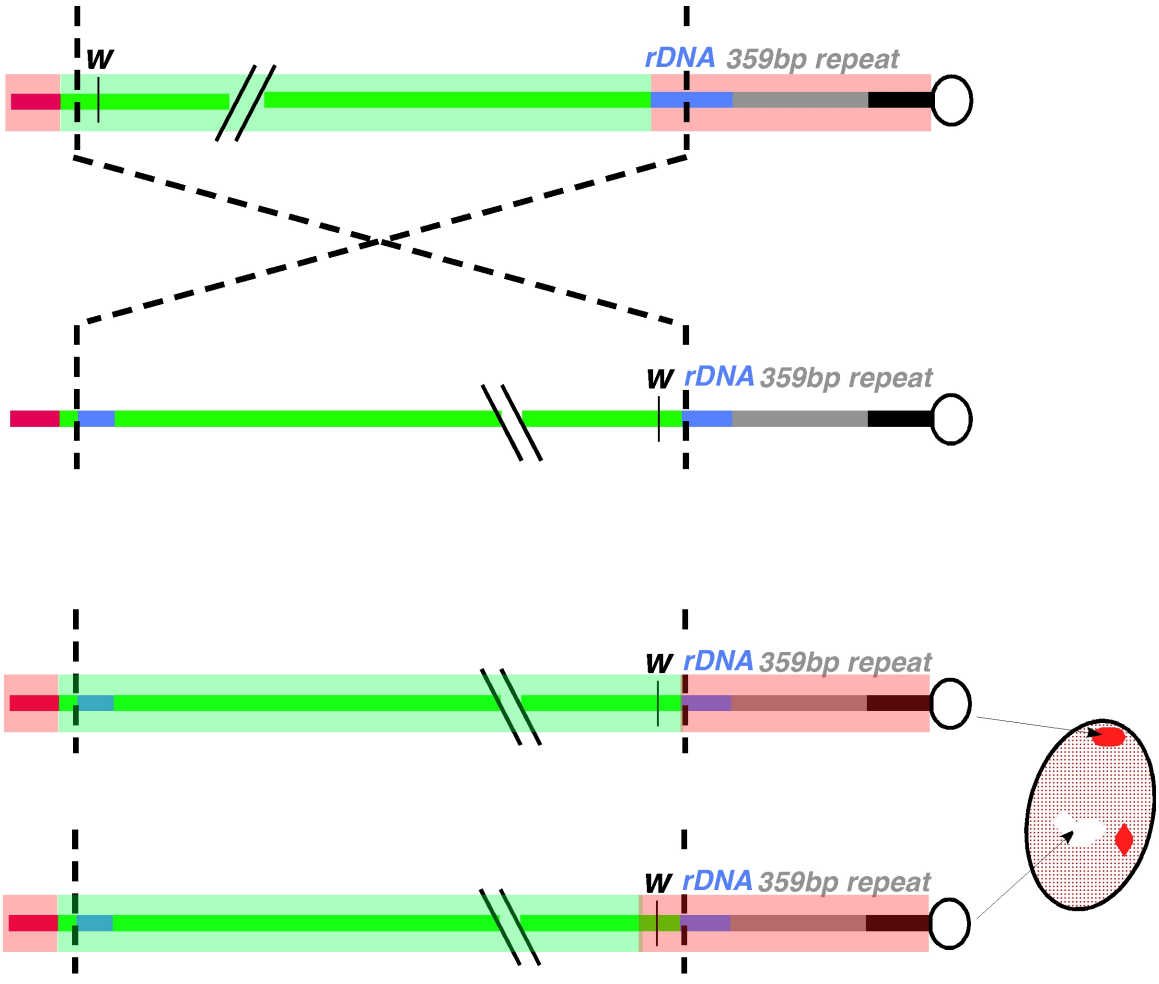
Chapter 1, Figure 1



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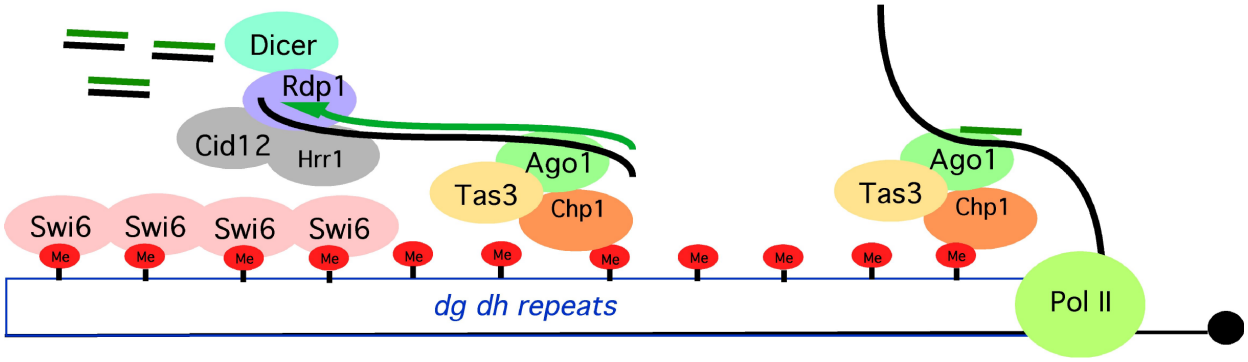
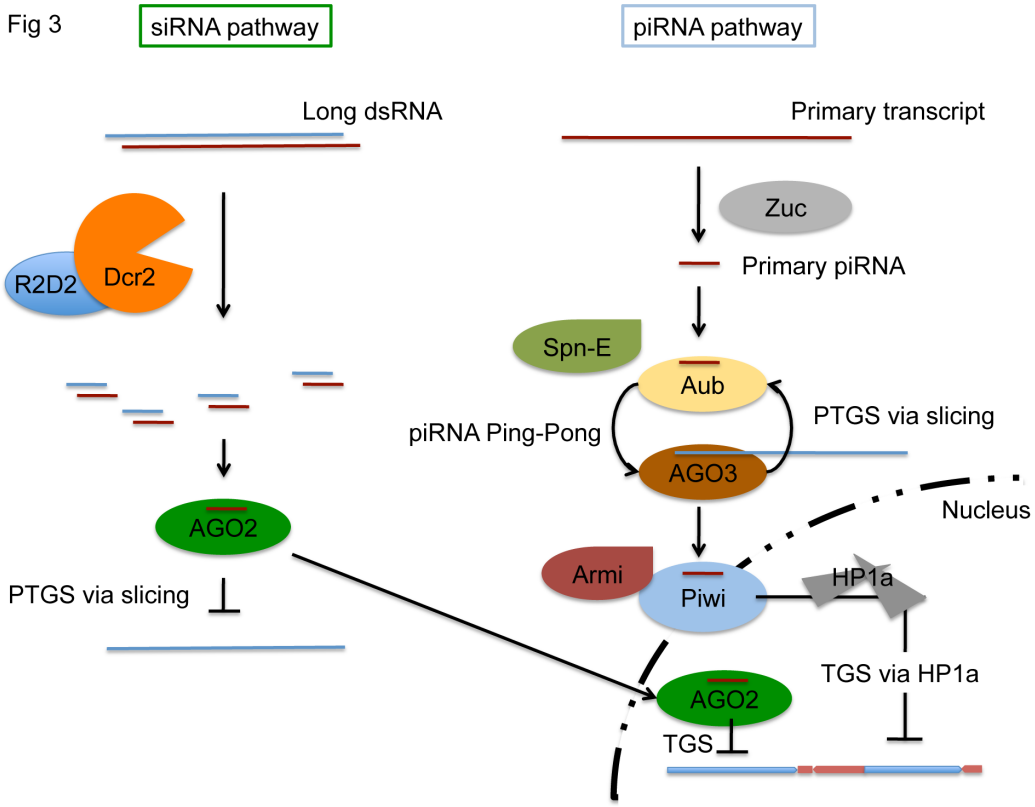


Chapter 1, Figure 3



Chapter 2

Fixation of the genetic background rather than an epigenetic state determines a PEV phenotype

Fixation of the genetic background rather than an epigenetic state determines a PEV phenotype

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Abstract

Chromatin-based transcriptional silencing is considered a stochastic process largely because of the mosaic expression observed in position effect variegation (PEV). Here we closely examine the impact of genetic background on PEV phenotypes. By consecutive generations of inbreeding, we isolate two inbred lines exhibiting contrasting degrees of variegation. Within each inbred population we observe remarkable similarity for both the degree and pattern of variegation. Further genetic analysis excluded the possibility that this result was due to fixation of epigenetic states, and indicates a strong genetic component in determining the phenotype. We propose a modified stochastic model to describe PEV. In this model, the genetic background and the position effect act together to determine the on/ off probability at the reporter locus for a given cell.

Introduction

Position effect variegation (PEV) describes the mosaic expression of a phenotype in a cell population that is otherwise uniform. It has generally been studied in cases where the cell-autonomous phenotype is easily visualized, such as eye pigmentation, but appears to be a general phenomenon (Tartof, 1994; Girton and Johansen, 2008). Muller reported the original observation of variegating eye pigmentation in adult flies, recovered following X-ray mutagenesis (Muller, 1930). Because of the high degree of variation in the

pattern and level of pigmentation between individuals and across generations, he described the phenotype as “ever sporting” (Muller, 1930). The report on this highly variable phenotype led to various speculative models describing how such a heritable, yet variable phenotype could arise (Spradling and Karpen, 1990). Further investigations have led to a transcriptional silencing model describing a stochastic spreading of heterochromatin (Tartof et al., 1984). The X-ray-induced inversion juxtaposed the *white* gene, which is required cell-autonomously for proper deposition of eye pigment, to the pericentric heterochromatin. The spreading of pericentric heterochromatin to the *white* locus, with concomitant silencing in some but not other cells, results in a variegated pattern of eye pigmentation. This spreading process is thought to be stochastic because no obvious rules can be derived from observed variegating patterns.

While the determination of the chromatin state in an individual cell appears to be stochastic, in many cases strong genetic components have been identified that impact the expression level of the phenotype for the cell population as a whole (Girton and Johansen, 2008). An assay for a PEV modifier activity has therefore been commonly used to determine the participation of a given gene of interest in the process of heterochromatin formation and gene silencing (Grigliatti, 1991). In fact, screens for PEV modifiers have been a major source in generating candidate genes for further analysis of the process of heterochromatin formation (Wustmann et al., 1989; Hayashi et al., 1990).

The PEV phenotype has also been utilized in a different way to further our understanding of heterochromatin. We previously devised a P element reporter to probe the heterochromatin landscape of the genome (Wallrath and Elgin, 1995). Using the well-characterized *hsp70* promoter to drive a *white* reporter gene, about 1 % of the insertion lines recovered following mobilization exhibited a variegating eye phenotype (Wallrath and Elgin, 1995). Mapping of these variegating insertion lines revealed an outline of heterochromatin distribution in the genome, confirming prior cytological assignments. PEV is observed following insertion of the reporter P element into the pericentric and telomeric regions of major autosomes and X chromosome, as well as unmapped regions of the Y chromosome. Based on the eye phenotype, the fourth chromosome (Muller F element) appears to be unique, with interspersed heterochromatic and permissive domains (Sun et al., 2000). Characterization of these variegating P element reporter insertion lines indicated the same basic mechanism for variegation as observed in the original *white* mottled line from Muller (sensitivity to sex chromosome dosage, temperature, etc.) (Wallrath and Elgin, 1995), although individual heterochromatic domains can show differences in sensitivity to a small subset of the known suppressors of variegation (Haynes et al., 2007; Brower-Toland et al., 2009).

Although PEV has been tremendously helpful in developing our understanding of heterochromatin, its stochastic nature has not been extensively examined. Numerous mutations have been identified that modify PEV; it is

estimated that there are ~150 modifiers in the fly genome (Schotta et al., 2003). Genetic background – including different assortment of alleles at these loci - could determine the probability for a spreading event to occur in a given fly within a stock, and thus could contribute to the variation seen in PEV phenotypes. Here, we look at the impact of genetic background on PEV by inbreeding a line with a P element reporter exhibiting variegation of *hsp70-white*. Consistent with a genetic determinant model, we find that the level of PEV is highly consistent among individuals and across generations in an inbred population. In contrast to a purely stochastic spreading model, the pattern of variegation displays a high degree of similarity between individuals in these lines, indicating a more controlled probability distribution for the spreading of heterochromatin amongst the cell population of each individual. Results with an independent variegating reporter (*LacZ*) verify that fixation of the phenotype is a consequence of the more consistent genetic background instead of fixation of epialleles at the reporter site. Our observations provide new insights into a classic system and suggest that quantitative trait mapping could be used to identify modifiers of PEV.

Results

To estimate the extent of the underlying genetic contribution to the variation of PEV between individual flies, we selected for extreme PEV phenotypes (pigment levels) in a given population in a controlled environment. The study was carried out using a 4^{th} chromosome PEV reporter line, 39C12, for

several reasons. First, we observed considerable variation in the levels of reporter expression in adult fly eyes between individuals in the population, albeit there was a genetic bottleneck in the production of this transgenic line. (The line is derived from a single male with the P element on the fourth chromosome, back-crossed to *y* w^{67c23} females.) Second, 39C12 is relatively well-characterized in terms of its response to PEV modifiers (Pal-Bhadra et al., 2004; Haynes et al., 2007; Brower-Toland et al., 2009). Third, its position has been mapped to a precise location in the genome (Sun et al., 2000). Finally, the *hsp70* promoter used in this reporter is well characterized (Weber and Gilmour, 1995). To select for extreme PEV phenotypes, we isolated the single female virgin displaying the strongest PEV eye phenotype from the parental population and mated it to a single male sibling with a matching eye phenotype. By repeating the process for 5 generations (full sibling mating followed by selection), one obtains a line (39C-12-A1) in which the level of eye pigmentation is low and appears to be rather uniform (Figure 1a). A weak PEV line, 39C-12-D1, was similarly derived (Figure 1a). Further generations of full sibling crosses do not enhance the consistency of eye phenotype between individuals to any greater degree (data not shown). While the fixation in the level of PEV suggests a strong genetic component in determination of this phenotype, as expected, we were surprised to see the consistency in the pattern of eye pigmentation among individuals within each inbred line (Figure 1b). This constancy suggests a more controlled mechanism in determining the PEV phenotype at the cellular level than a purely stochastic spreading model would suggest.

The fixation of phenotype by inbreeding suggests that the level of expression and pattern in PEV are strongly determined by genotype. To further test this idea, we performed crosses between the two inbred lines to generate F1 and F2 populations. Assuming a genetic determinant model for the heritability of this phenotype (rather than an epigenetic model), we expect to see an F1 population with a uniform PEV phenotype and an F2 population with a wide spectrum of PEV phenotypes resulting from meiotic recombination between the A1 and D1 haplotypes. This result is obtained from crosses in both directions (Fig 2 a, b). The average pigmentation level for F1 progeny from both crosses falls right in the middle between the pigment levels of the parental A1 and D1 lines, with a slight increase in variance compare to either the parental line (Fig 1a, 2a; Supp Fig 1). While the mean pigmentation level for the F2 progeny remains in a similar range as for the F1 progeny, a huge increase in variation of the PEV phenotype between individuals is observed (Fig 2 a, c). The range of phenotypic variation in the F2 population resembles that of the starting 39C12 stock (compare Fig1a with 2a). These results support a genetic determinant model for the heritability of PEV. The distribution of PEV phenotypes in the F1 and F2 populations further suggests that there are multiple modifiers in both the A1 and D1 haplotypes.

While these observations can be adequately explained by effects from background PEV modifiers, there exists a formal possibility that all of the

observed phenomena result from fixation of alternative epigenetic states on the PEV reporter itself. This alternative is attractive, given that the starting line (39C12) was derived from a single male crossed with a standard stock, and should therefore be relatively homogeneous, and the fact that PEV is known to reflect epigenetic phenomena. In an epigenetic model, one would envision a mechanism by which inbreeding leads to fixation of a chromatin state, which results in a uniform PEV phenotype within the population. The fixed epigenetic state on the reporter would be relaxed in crosses between lines of different epigenetic states, explaining the slight increase in the phenotypic variation observed in the F1 population. In the F2 population, the two further relaxed epi-alleles would randomly segregate, leading to a strong increase in phenotypic variation in the F2 population. While this model is purely speculative, it provides an interesting way to think about heritability of a phenotype.

To distinguish between the genetic and epigenetic models, we introduced a different PEV reporter into the A1 or the D1 genetic background. We utilized dominantly marked balancer chromosomes [and the fact that meiotic recombination does not occur in the male germ line (Hess and Meyer, 1968; Orr-Weaver, 1995)] to introduce a Y-linked PEV reporter without perturbing the genetic background. The newly introduced PEV reporter will not be in a fixed epigenetic state, and we can therefore examine the effect of the background genetic modifiers on the PEV phenotype. The PEV phenotype of the BL2 *LacZ* reporter line used for this purpose is resulted from a translocation of the reporter

to the Y chromosome following X-ray irradiation (Lu et al., 1996). The level of beta-galactosidase activity in lysate prepared from single male fly has been used as readout for the PEV phenotype (silencing, loss of activity). We observe a uniform PEV phenotype for the BL2 reporter in either the A1 or D1 genetic backgrounds, with D1 flies exhibiting ~5 times the activity of A1 flies (Fig 3). This result strongly supports the genetic determinant model over the epigenetic state model. The difference in the level of PEV for the BL2 reporter in the two genetic backgrounds is similar to what is observed for the 39C12 reporter. These results indicate that not only is the PEV phenotype strongly determined by the background modifiers, but also that similar impacts are exerted by the same set of background modifiers on two very distinct reporters. We therefore infer that a shared pool of factors is impacting 4^{th} chromosome and Y chromosome heterochromatic silencing.

Discussion

PEV has been considered to be a highly variable phenotype, and its variability is commonly attributed to its epigenetic component. By applying consecutive rounds of selection, we were able to generate two populations having a uniform PEV phenotype with contrasting degrees in variegation. For the reporter lines tested in this study, both the degree and pattern of PEV appears to be largely determined by the genotype. Our results indicate that the degree of variegation is a quantitative trait; thus established methods of population genetics

could be used to identify causative quantitative trait loci impacting this phenotype. Given the ease in generating large segregant populations in flies, and the decreasing cost of next-generation sequencing technology for genotyping, this should be promising. Such an approach is likely to generate informative results that will help us understand the chromatin-based transcriptional silencing mechanism, which is emblematic for the PEV phenotype.

The slight increase in variability in the PEV phenotype amongst the F1 population of a cross between the two inbred lines raises questions concerning the genetic determinant model for PEV. The prevalent view of PEV being an epigenetic phenomenon forced us to consider the formal possibility of fixing epi-alleles by full sibling crosses. The lack of knowledge in the field on how “epi-alleles” would transmit across generations makes it difficult to experimentally distinguish between the two alternatives. Assuming that the epigenetic state of an epi-allele will be linked with the reporter itself, we choose to swap in an entirely different PEV reporter at a different genomic locus in order to reject the epi-allele hypothesis. Our results clearly demonstrate that the fixation of PEV phenotypes in the population by inbreeding is not linked to the reporter locus. This observation provides a strong argument supporting a genetic determinant model. In addition, on substituting a Y chromosome reporter into the A1 and D1 genetic background we observed the same direction of impact on reporter expression as was seen for the 4^{th} chromosome 39C12 reporter. This observation indicates that a common set of machinery operating in

heterochromatin formation/ maintenance is likely shared between the 4th chromosome and the Y chromosome. We have previously reported on the unique properties of 4th chromosome heterochromatin, particularly on contrasting PEV response to some modifiers that are known to be critical to pericentric heterochromatin (Haynes et al., 2007). Further investigations of Y-linked PEV will likely reveal interesting feature of Y chromosome heterochromatin.

Based on the current model for PEV, the high degree of resemblance of eye pigmentation patterns observed between individuals in each inbred population is unexpected. The difficulty of quantifying similarity of pigmentation patterns between individuals hinders a thorough investigation on the impact of genetic variation on this trait. Nonetheless, by visual inspection, it is apparent that for reporter 39C12 in either the A1 or D1 background, the PEV pattern tends to have more concentrated pigmentation in the posterior quadrant of the eye (Figure 1b). This pattern is not observed for the BL2 reporter in those respective genetic backgrounds (Figure 3). It appears that for the 39C12 insertion site, the posterior quadrant of the eye is more permissive for an open chromatin structure and therefore the expression of eye pigmentation. We propose that the genetic background and the genomic locus of the reporter insertion site together determine the probability for each ommatidium pigment cell to adopt an open or closed chromatin structure at the reporter site. While the genetic background sets up a general probability for heterochromatinization as defined by variants in trans-acting factors, the position effect of cis-acting elements could further

determine the differential probability seen in different areas of the fly eye. This modified stochastic model describes the overall similarity and the subtle variations of eye pigmentation between individuals in the inbred population. It remains to be determined as to how generally applicable this model is to other genomic loci.

Materials and Methods

Fly husbandry and genetics

Flies are cultured at 25 C, 70% humidity on regular cornmeal sucrose-based medium (Shaffer et al., 1994). The 39C12 reporter line (Wallrath and Elgin, 1995) was used as the starting line to generate A1 and D1 inbred lines. Consecutive full sibling crosses with selection for extreme eye phenotype at each generation were performed to create the two inbred lines. Eye phenotype appears to be fixed after 5 generations of full sibling crosses. To substitute the BL2 (Lu et al., 1996) Y-linked PEV reporter into the A1 and D1 genetic background, we used dominantly marked balancers to follow the second and third chromosomes. Balancers were first introduced to the BL2 line by a standard cross and the male progeny that had both second and third chromosomes balanced were selected to mate with female flies from the inbred line. Male progeny from this cross with both balancers over inbred chromosomes were backcrossed to the inbred line. Progeny from the backcross lacking both balancers were selected to make the

stock. This stock was made homozygous for the 4th chromosome, as judged by the absence of the 39C12 reporter in the adult female progeny, to generate the final stock.

PEV assays

Eye pigment extraction and quantification was done essentially as previously described (Sun et al., 2004) with a few modifications. Instead of hand homogenizing for pigment extraction, a Mixer Mill Mm 300 was utilized to increase the throughput and consistency. The overnight incubation at 4°C was then omitted. For each genotype 20~30 males were randomly selected from the population and sorted according to their pigmentation level by visual inspection. Five flies of similar pigmentation levels were than collected together as one sample.

X-gal staining of eye imaginal discs and the assay of beta–galactosidase activity were done as previously described (Sullivan et al., 2000).

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Figure legend

Figure 1. Selective inbreeding results in fixation of the PEV phenotype. (a) Quantitative assessment of pigment levels in the adult fly eye representing the degree of PEV. Each data point represents a reading from samples of the indicated genotype, parental (39C12) or selected (A1, D1) (see Materials and

Methods for details). *yw* is used to indicate the background signal level. (b) Images of the PEV pattern in the adult fly eye taken from randomly selected individuals in each A1 and D1 inbred populations.

Figure 2. PEV phenotype of the progeny from the cross between the A1 and D1 inbred lines. (a) PEV levels in the adult progeny. Each data point represents a sample of the indicated genotype. Results observed were essentially the same from crosses in either direction. (b) The PEV pattern in the adult fly eye from randomly selected individuals from among the F1 progeny of a cross between the A1 and D1 inbred lines in the indicated direction. (c) Selected images of the PEV pattern in the F2 population representative of the diversity in pigmentation levels observed.

Figure 3. PEV phenotype of the Y-linked BL2 reporter in the A1 and D1 genetic backgrounds. The level of PEV is quantified by measuring the activity of the beta-galactosidase reporter gene. Each bar represents the activity level measured in lysate prepared from a single adult fly of the indicated genotype. Representative images of eye pigmentation for each genotype are shown below the bar graph.

Fig 1a

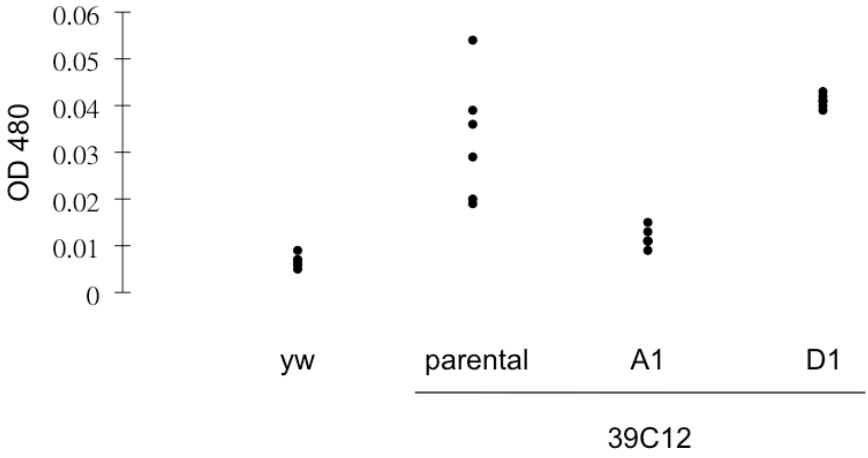


Fig 1b

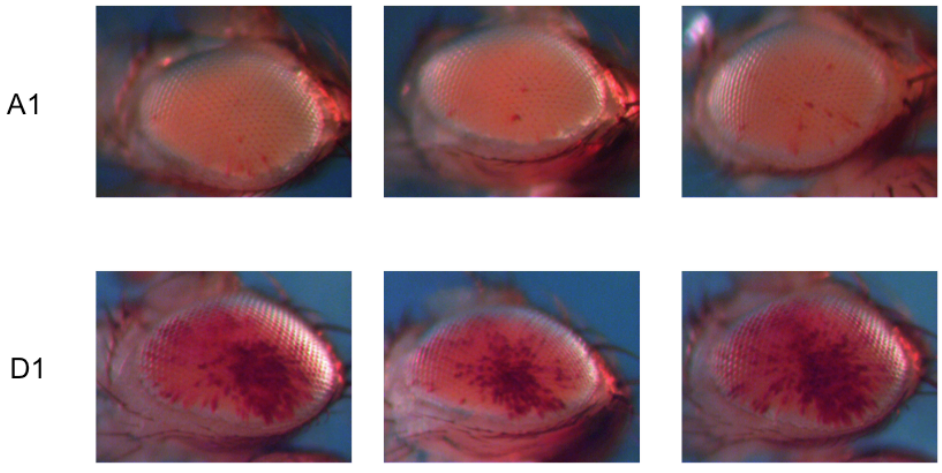


Fig 2a

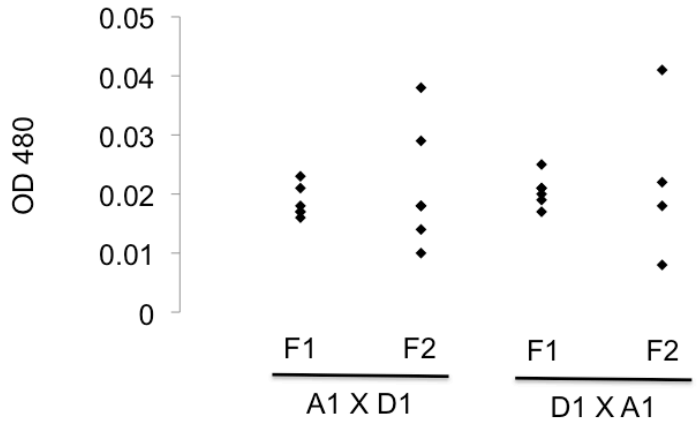


Fig 2b

Female X male

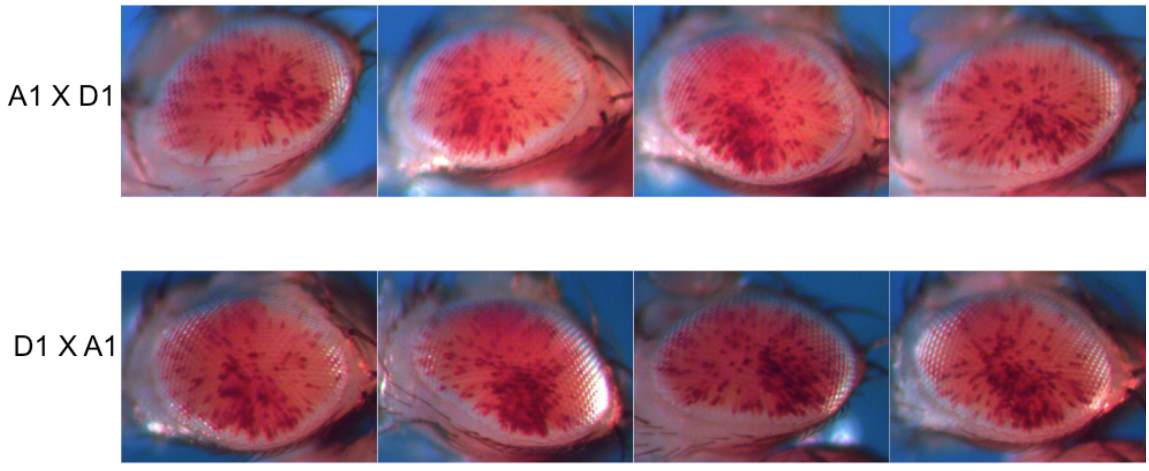


Fig 2c

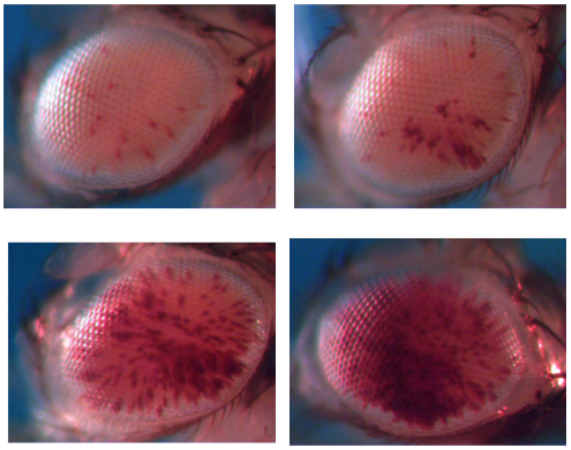
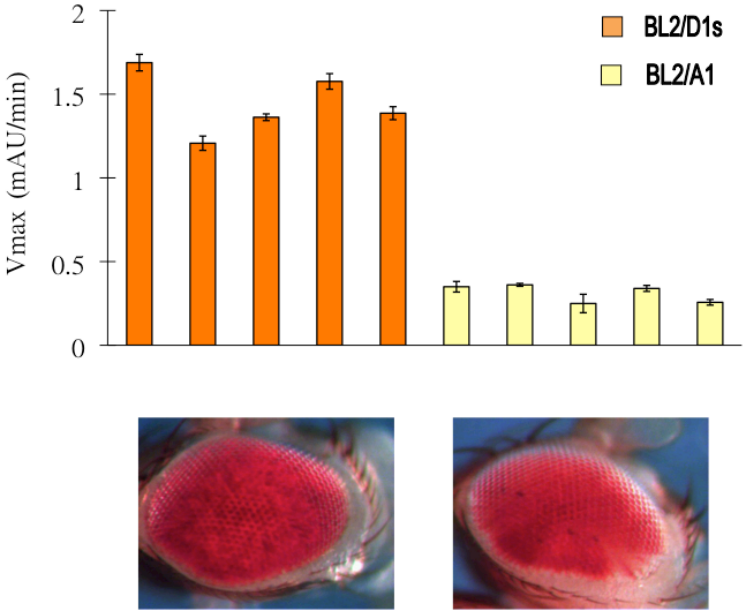
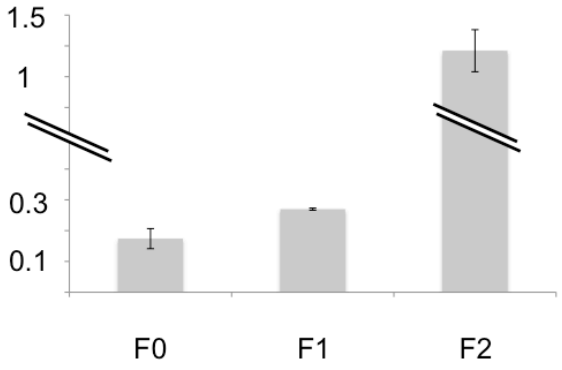


Fig 3



Supp fig 1



Standard deviation of pigment level in each generation from a cross between inbred line

Chapter 3

A Distinct Type of Heterochromatin at the Telomeric Region of the *Drosophila melanogaster* Y chromosome

Contributions to the manuscript

Work presented in this chapter is a collaborative effort from all coauthors. Drosophila lines carrying the *hsp70-white* reporter on the Y chromosome were previously recovered in the Elgin lab, as described in Sun et al. (2004) Mol Cell Biol 24: 8210-20, Riddle et al. (2008) Genetics 178: 1177-91, and Sentmanat and Elgin (2012) Proc Natl Acad Sci USA, in press. Under the supervision of SCRE, I initiated the project by performing a preliminary analysis of the impact of PEV modifier mutations on two of the eight Y-linked reporter lines. I also did inverse PCR mapping of all eight Y-linked insertions. *Our collaborators, Patrizio Dimitri and Maria C Accardo, did in situ hybridization mapping of the inserts on mitotic chromosomes*. Ruth Nan carried out most of the extended mutant analyses under my supervision. As the first author, I interpreted the data and wrote the manuscript with input from SCRE and other members of the Elgin lab.

A Distinct Type of Heterochromatin at the Telomeric Region of the *Drosophila melanogaster* Y chromosome

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Abstract

Heterochromatin assembly and its associated phenotype, position effect variegation (PEV), provide an informative system to study chromatin structure and genome packaging. In the fruit fly *Drosophila melanogaster*, the Y chromosome is entirely heterochromatic in all cell types except the male germline; as such, Y chromosome dosage is a potent modifier of PEV. However, neither Y heterochromatin composition, nor its assembly, have been carefully studied. Here, we report the mapping and characterization of eight reporter lines that show male-specific PEV. In all eight cases, the reporter insertion sites lie in the telomeric transposon array (HeT-A and TART-B2 homologous repeats) of the Y chromosome short arm (Ys). Investigations of the impact on the PEV phenotype of mutations in known heterochromatin proteins (i.e. modifiers of PEV) show that the Ys telomeric region is a unique heterochromatin domain: it displays sensitivity to mutations in HP1a and to depletion of EGG and SU(VAR)3-9, but no sensitivity to *Su(z)2* mutations, It appears that the endo-siRNA pathway has a major targeting role for this domain, but that an ectopic copy of *1360* is sufficient to induce a piRNA targeting mechanism to further enhance silencing. These results demonstrate the diversity of heterochromatin domains, and the corresponding variation in targeting mechanisms.

Introduction

Heterochromatin represents a unique type of chromatin structure that confers transcriptional silencing by regular packaging of distinct domains enriched for repetitious DNA (Elgin, 1996). Abnormalities in the formation and/or maintenance of heterochromatin therefore are commonly associated with transposon activation and genome instability (Peng and Karpen, 2008). In addition, heterochromatin also plays an importance role in cell division; as part of the centromeric structure, heterochromatin is critical for proper segregation of chromosomes during mitosis (Dalal et al., 2007). Other regulatory roles of heterochromatin, such as telomere length homeostasis (Schoeftner and Blasco, 2009) and proper expression of heterochromatic genes (Yasuhara and Wakimoto, 2006), have also been documented.

The Position Effect Variegation (PEV) phenotype, commonly monitored in the adult fly eye, has been used in many previous studies as an indicator of the degree of heterochromatin formation at the underlying locus (Girton and Johansen, 2008). PEV results from positioning a euchromatic reporter gene in or close to a heterochromatic environment by rearrangement or transposition. Several lines of evidence indicate that the spreading of heterochromatin packaging into the promoter region of the euchromatic gene is the cause of transcriptional silencing (Tartof et al., 1984; Girton and Johansen, 2008). Silencing of the underlying euchromatic gene occurs in some but not all cells in a

population, giving rise to a variegating phenotype; this differential spreading of heterochromatin is suggested to be a stochastic process (Cheutin et al., 2004). The extent of silencing (variegation) can serve as a proxy for the degree of heterochromatin formation at the particular locus. A mutation that impacts the level of PEV is therefore indicative of a gene that functions in the formation and/or maintenance of heterochromatin (Schotta et al., 2003). Identification of mutations resulting in strong suppression of PEV (loss of silencing) and molecular characterization of these *Su(var)* loci has laid the groundwork for understanding heterochromatin formation in flies (Wustmann et al., 1989; Grigliatti, 1991). Genes such as *Su(var)3-9* (a histone H3K9 methyltransferase) and *Su(var)3-3* (an H3K4 demethylase), identified and characterized under this paradigm, have revealed much of what we know about heterochromatin (Girton and Johansen, 2008).

PEV has been used as an assay to probe the heterochromatic landscape of the genome (Wallrath and Elgin, 1995). A P-element harboring an *hsp70-white* reporter gene was mobilized in the fly genome to identify heterochromatic regions, those that induce a variegating eye phenotype (Wallrath and Elgin, 1995). This screen recovered lines with insertions into pericentric domains, telomeres (TAS elements), the fourth and the Y chromosome, as anticipated from prior cytogenetic analysis. Thus, it produced PEV reporter lines that can be used to monitor the structure of heterochromatin across the genome. Use of these lines quickly established that not all heterochromatin has the same

composition; different domains show distinct responses to different *Su(var)* mutations (Cryderman et al., 1999; Haynes et al., 2007). Based on the differential response profile to well-known suppressors of variegation, a major distinction has been made between pericentric and telomeric heterochromatin, suggesting different mechanisms are involved (Doheny et al., 2008). In particular, telomere position effect (TPE; studied using reporters in the TAS elements) is inert to mutations in *Su(var)205* (which codes for HP1a) but is suppressed by mutations in *Su(z)2* (a component of the Pc system) (Cryderman et al., 1999; Doheny et al., 2008), while the opposite is true for pericentric PEV. Further analyses have revealed more unique domains of heterochromatin in the genome. For example, fourth chromosome PEV is not generally suppressed by mutations in *Su(var)3-9* (Haynes et al., 2007), but is sensitive to mutations in *egg*, indicating that a different HMT is required for silencing (Tzeng et al., 2007; Seum et al, 2007b; Brower-Toland et al., 2009). Additional analysis of this type is likely to reveal more distinct types of heterochromatin, presumably reflecting differences in the underlying DNA repeat sequences.

The question of how different types of heterochromatin are established in the genome remains an active area of research. Different targeting mechanisms could be utilized for different types of heterochromatic domains. In flies, both the piRNA pathway and the endo-siRNA pathway have been implicated in targeting heterochromatin formation (Brower-Toland et al., 2007; Fagegaltier et al., 2009; Wang and Elgin, 2011). While these studies provide evidence supporting a small

RNA targeting model for heterochromatin formation at some repetitious elements, given the diversity of heterochromatin domains, one can also anticipate a diversity of targeting mechanisms.

In flies, the Y chromosome has long been known to be a largely heterochromatic domain. In fact, Y chromosome dosage was one of the first modifiers of PEV to be identified (Gowen and Gay, 1934). The Y has been described as a ‘sink’ for components essential for heterochromatin formation and/or maintenance. It appears that additional copies of the Y chromosome impact PEV at other loci in the genome by competing for a limited amount of key factors required for heterochromatin integrity (Locke et al., 1988; Dimitri and Pisano, 1989). Recently, reports from Hartl and colleagues have further elaborated on how polymorphism in Y chromosome heterochromatin can impact chromatin-based regulatory processes in other regions of the genome (Lemos et al., 2010; Zhou et al., 2012). However, relatively little is known about the formation / maintenance of heterochromatin on the Y chromosome itself. A paucity of PEV reporter lines for the Y chromosome is one of the major obstacles in studying the mechanisms involved in heterochromatin formation and maintenance within this domain.

Over the past decade, we have collected eight variegating lines exhibiting a male-specific PEV phenotype. Here we map the insertion sites of these eight lines to the telomeric transposon array (HeT-A and TART-B2 repeats) of Ys

(short arm of the Y chromosome). We further characterize the heterochromatic properties of this region by examining the impact of mutations in PEV modifiers on these reporters. While the telomeric Ys heterochromatin shows a unique response profile compared to other parts of the genome, our studies nonetheless suggest that some of the mechanisms for heterochromatin formation and maintenance are shared among the Y chromosome, pericentric, and 4th chromosome heterochromatin. While it appears that the endo-siRNA pathway is likely the major mechanism used to target heterochromatin formation at this domain, an ectopic copy of the *1360* transposon remnant is sufficient to drive a piRNA-dependent heterochromatin targeting mechanism to further enhance silencing. We conclude that the Ys telomeric region is a unique domain of heterochromatin; further investigation of this region will be informative in understanding chromatin packaging in general.

Results

To precisely locate the insertion sites of the Y-linked PEV reporters, we performed inverse PCR followed by sequencing. While we cannot precisely map the location of each insert using BLASTN against the entire genome assembly, we can nonetheless map the insertion sites for all eight Y-linked reporter lines in internal regions of the telomeric retrotransposons. In six of these lines the reporter element is inserted into a TART-B2 element, while in the other two lines (39C66 and 8M112) the reporter is inserted into a HeT-A element (HeT-A

subfamily D and HeT-A to HeT-A Junction respectively). Surprisingly, all six reporters inserted into the TART-B2 element are located in the 3'-UTR of the element, with five of them clustered within a 50 nts range when mapped back to a TART-B2 consensus sequence (Fig1a). Because there are multiple copies of TART-B2 elements on the Y chromosome, and the quality of this region of the published assembly is relatively poor, we cannot resolve in which TART-B2 elements these insertions reside. However, a comparison of the flanking sequences among the six insertion lines identifies sequence polymorphisms, which suggests that the inserts are in different copies of TART-B2. This result suggests that there is a common region in TART-B2 3'UTR that is a particular hotspot for P-element insertions, consistent with results previously described by Mason and colleagues (Biessmann et al., 2005).

HeT-A and TART elements are distributed throughout telomeric and pericentric regions of the Y chromosome (Berloco et al., 2005). To further distinguish between these potential insertion sites, we performed *in situ* hybridization on metaphase chromosomes using the reporter sequence as a probe. Interestingly, we found that all eight lines are inserted in the tip of Ys (Fig 1b). These observations allow us to conclude that all eight reporter lines characterized here have an insert in the telomeric terminal retrotransposon array of Ys. The cytological results are consistent with the molecular mapping results presented above – both indicate that this region of the Y chromosome is relatively accessible to P element insertions (Fig 1). It should be noted that

reporters inserted into the terminal retrotransposon array have been previously described for the major autosomes (Biessmann et al., 2005). It has been reported that most of these insertion lines do not show a variegating phenotype, unless the reporter is located close to the TAS region (Biessmann et al., 2005). We therefore infer that our variegating reporters likely reflect an equilibrium between the spreading of adjacent heterochromatin and the expression of these retrotransposons.

These variegating reporter lines present a unique opportunity to elucidate the chromatin structure at a telomere of the Y chromosome. We first looked at the response of these variegating reporters to well-characterized modifiers of PEV and TPE (Fig 2). Given that all of these reporter lines carry inserts in the HeT-A and TART elements of Ys, we chose two lines, 39C66 (a HeT-A insert) and 8M76 (a TART insert), as representatives of this set for further analysis. We first tested dominant effects of mutations in modifiers of TPE. Multiple alleles of *Su(z)2*, a transcription factor, have previously been shown to significantly suppress TPE of TAS inserts (Cryderman et al, 1999; Doheny et al., 2008). We therefore tested its impact on our reporter lines. No obvious suppression effects were observed for both of the alleles tested here (Fig 2a), suggesting that the telomeric retrotransposon region of Ys does not have a chromatin structure typical to the TAS telomeric arrays, which are immediately upstream of HeT-A and TART arrays in the autosomes. We next examined the impact of a classic PEV suppressor, *Su(var)205*, on these reporters. *Su(var)205* encodes a chromo-

domain containing protein, HP1a, which is implicated in the formation and spreading of heterochromatin (James and Elgin, 1986; James et al., 1989; Eissenberg et al., 1990). Despite the known role of HP1a in telomere capping (Cenci et al., 2003; Perrini et al., 2004), mutations in *Su(var)205* do not appear to modify TPE as seen using lines with the reporter inserted in TAS (Cryderman et al., 1999). Interestingly, despite the fact that inserts are found in the telomeric region, both alleles tested here for *Su(var)205* show significant dominant suppression of variegation for both reporters (Fig 2a, b). These observations suggest a response profile for the Ys HeT-A/TART telomeric heterochromatin, which is more similar to PEV than TPE. We next tested the impact of an insertion mutation of *Su(var)3-9*. The *Su(var)3-9*^{06} allele disrupts the production of the SU(VAR)3-9 protein (Ebert et al., 2004) and has been shown to impact both TPE and PEV (Doheny et al., 2008). Strong dominant suppression of variegation is observed with this allele for these Y-linked reporters (Fig 2a, b), indicating an important role for this gene product in the chromatin structure at this region.

SU(VAR)3-9 is a histone 3 lysine 9 methyl-transferase. Given the strong impact of the *Su(var)3-9*^{06} allele on variegation, we reasoned that SU(VAR)3-9 might function through its enzymatic activity to modify the chromatin structure at this region. However, an allele disrupting the enzymatic activity of SU(VAR)3-9 (Ebert et al., 2004), *Su(var)3-9*^{02}, did not show a suppression of variegation (Fig 3a). This suggests that the critical function of SU(VAR)3-9 in this region is structural rather than enzymatic. This interpretation is supported by previous

results documenting the antipodal effect of SU(VAR)3-9 on PEV (Locke et al., 1988). Our observations lead us to infer that SU(VAR)3-9 is likely not the HMT functioning in the Ys telomeric heterochromatin.

To identify the potential HMTs functioning at this region, we looked for dominant effects from mutations in the genes for other known HMTs. In addition to *Su(var)3-9*, two more genes, *egg* and *G9a*, have been identified in the fly genome as potential HMTs (Stabell et al., 2006a, 2006b; Seum et al., 2007a, 2007b). Dominant effects of mutations in *egg* and a recessive effect of *G9a* were tested for their impact on the variegation phenotype of these reporters. Only the $egg^{1473.8}$ allele shows a strong suppression of variegation at these sites, consistent with the interpretation that EGG is the major HMT functioning in the formation/ maintenance of heterochromatin at the Ys telomeric region. The two *egg* alleles tested show different effects on the suppression of variegation. The $egg^{1473.8}$ allele is a deletion of the entire SET domain, a domain which is required for the HMT activity of EGG (Clough et al., 2007). In contrast, the egg^{235} allele has a di-nucleotide substitution that creates a cryptic splice site for the 4th intron (Clough et al., 2007). Retention of this intron will introduce a premature stop codon that results in a protein product with no identifiable functional domains. However, a cryptic splice site actually allows normal splicing to occur at a low frequency, which results in the production of some wild type protein (Clough et al., 2007). The comparison on the impact from the two *egg* alleles therefore represents a comparison between a dominant effect of the SET domain deletion

and an incomplete null mutation. We interpret the discrepancy between the two alleles in their impact on variegation as an additional piece of evidence demonstrating the importance of the HMT activity of EGG in this region.

EGG has previously been characterized as a 4th chromosome-specific HMT (Seum et al., 2007b; Tzeng et al., 2007; Brower-Toland et al., 2009). It is also known to impact expression from some reporters in the pericentric heterochromatin (Brower-Toland et al 2009). Nonetheless, the observations above suggest that the 4th chromosome and Ys telomeric heterochromatin share common components for heterochromatin formation or maintenance. To test this hypothesis, we took advantage of the attached 4th chromosome line (Haynes et al., 2007) to generate flies with only one copy or with three copies of the 4th chromosome to examine the impact of dosage on Ys telomeric heterochromatin. Increasing heterochromatic mass of a particular type in the genome could lead to increased competition for the available components for heterochromatin formation/ maintenance (Locke et al., 1988). On increasing dosage of the 4th chromosome, we observed a suppression of variegation for the Y-linked reporters (Fig 3a). Previous studies have shown that Y chromosome dosage has a similar impact on fourth chromosome reporters (Wallrath and Elgin, 1995). These observations reinforce the conclusion that the 4th chromosome and the Ys telomeric heterochromatin share some components for heterochromatin formation and/or maintenance.

Small RNA targeting mechanisms have been demonstrated to be one of the major mechanisms for initiating the formation of heterochromatin in the fission yeast *S. pombe* (Volpe et al., 2002; Verdel et al., 2004). In the fruit fly, both siRNA and piRNA systems have been implicated in this process (Pal-Bhadra et al, 2004; Brower-Toland et al., 2007; Fagegaltier et al., 2009; Wang and Elgin, 2011). To ask whether small RNA targeting of heterochromatin formation could participate in the formation of Ys telomeric heterochromatin, we examined the impacts of dominant mutations in both siRNA and piRNA pathways. No obvious impact is observed when mutations in components of the piRNA pathway are introduced (Fig 4a, b). We examined the impacts of $piwi^{1}$, $piwi^{2}$, aub^{QC42} and hls^{125} mutations on the variegation of the Ys telomeric reporters, and no suppression effects were observed (Fig 4a). In contrast, both alleles we tested mutating components of the siRNA pathway strongly suppress variegation (Fig 4a,b), indicating an involvement of this pathway in the heterochromatin silencing of the Y chromosome. $Dcr\text{-}2^{R416X}$ has a point mutation that truncates the protein produced and disrupts its function in producing siRNA (Lee et al., 2004). $ago2^{414}$ is a loss of function allele with its second exon deleted by imprecise excision (Okamura et al., 2004). That mutations in the siRNA pathway dominantly suppress variegation indicates a role for siRNA in targeting heterochromatin formation in this region.

Transposon density, particularly that of the *1360* DNA transposon, has been shown to be correlated with heterochromatin silencing on the 4th

chromosome (Sun et al., 2004). Previously we have shown that introducing an extra copy of *1360* can enhance the variegation phenotype in regions sensitive to mutations in small RNA pathways (Haynes et al., 2006). In an independent screen using a reporter containing a copy of *1360* upstream of the *hsp70-white* reporter (Fig 5a), we recovered an additional Y-linked PEV line, line 1250. The *1360* element in this construct is flanked by FTR sites, which allows FLP-mediated excision to test its impact on PEV (Fig 5a). We were unable to precisely map the insertion site of line 1250 using inverse PCR sequencing. However, *in situ* hybridization experiments map the insertion site again in the telomeric region of Ys (Fig 5b). Reporter line 1250 therefore provides an opportunity to examine the sensitivity of variegation in the Ys telomeric region to an ectopic *1360* element. FLP-mediated excision of the *1360* element resulted in strong suppression of variegation at this locus (Fig 5 c, d), consistent with the interpretation that the exogenous *1360* element plays an important role in promoting local heterochromatin structure. We next investigated the potential mechanism of this *1360*-dependent enhancement of heterochromatin silencing. We examined the impact of mutations in the siRNA and piRNA pathways on the variegation phenotype in reporter lines 1250 with and without the extra copy of the *1360* element. In the absence of the extra *1360* element, the reporter line 1250 shows similar responses to mutations in components of the small RNA pathways as seen for the other lines tested in this study (compare Fig 4a, 5c). Interestingly, with the ectopic copy of *1360* element present, the same reporter appears to show dominant suppression of variegation in response to mutations of

piwi and *aub* (Fig 5c). This suggests that the enhancement of variegation resulting from the extra copy of the *1360* element is operating via a piRNA dependent targeting mechanism. We therefore conclude that the ectopic copy of the *1360* element at the Ys telomeric region is sufficient to recruit the piRNA-dependent targeting machinery to enhance heterochromatin silencing at a locus that is normally dependent on the siRNA pathway for heterochromatin targeting.

Discussion

Despite being one of the first heterochromatic regions in the fly genome to be identified, the packaging of Y chromosome is not well understood. The poor quality of the sequence assembly in this region of the fly genome severely restricts our ability to perform a comprehensive survey of its chromatin landscape. Reporter insertion lines that can be uniquely mapped by *in situ* hybridization therefore present unique opportunities to explore the chromatin packaging of this region.

Screens using a P element carrying an *hsp70-white* reporter have led to the recovery of variegating lines with an insertion into the HeT-A/TART arrays at the Ys telomere. This is surprising, in that the HeT-A/TART retrotransposons are know to be expressed, and prior studies (Biessman et al 2005) have reported that insertions into similar telomeric sequences in the autosomes results in full expression, unless the reporter is positioned close to the proximal TAS arrays,

which are silenced. We have analyzed the impacts on the observed PEV phenotype of our Ys telomere reporters resulting from mutations in PEV modifiers that are well characterized. We have found that these reporters do not mimic the TAS-associated TPE response; instead they show strong suppression in response to mutations in HP1a. In addition, we found that while the chromatin structure at this region is sensitive to the dosage of Su(var)3-9 (in contrast to 4^{th} chromosome heterochromatin), it actually requires the SET domain of EGG for proper silencing (similar to 4^{th} chromosome). These results enable us to conclude that the telomeric Ys is a unique domain of heterochromatin. Whether this reflects a structure uniquely targeted to these Y chromosome HeT-A and TART elements, or the spreading of a heterochromatin structure targeted to other adjacent repetitious elements, cannot be determined given the current information. Regardless, we proposed that the Ys telomeric region should be added to the list of distinct subcategories of heterochromatin. While each of these domains has unique characteristics, they nonetheless appear to share certain modifiers and utilize some of the same proteins for heterochromatin formation.

Given the repetitive nature of Y chromosome, we proposed a targeting mechanism for its heterochromatin formation that utilizes small RNAs derived from transposable elements. We found that while reporters in this region normally respond to mutations in the endo-siRNA pathway, an ectopic copy of the1360 element is sufficient to enhance heterochromatic silencing via a piRNA-

dependent silencing pathway. This observation again suggests complex cross talk between different mechanisms of heterochromatin targeting/ formation.

Our identification of an additional type of heterochromatin corroborates the multiple chromatin states models resulting from large-scale genome wide studies of the distribution of histone modifications and chromosomal proteins, such as modENCODE (Kharchenko et al., 2011). As our study demonstrates, while the heterochromatin / euchromatin dichotomy is useful and convenient in describing much of what we know about chromatin structure, it is inadequate in capturing the diversity of chromatin structures within a genome. Future studies on the Y chromosome heterochromatin will likely yield new insights on the process of chromatin packaging and gene regulation.

Materials and Methods

Fly stocks, genetics and husbandry

Fly lines 39C66, 5M298, 7M27, 7M143, 7M415, 8M76, 8M112 and 8M114 were recovered from transposition-based screens that have been previously reported (Wallrath and Elgin, 1995; Riddle et al., 2008). Crosses testing for a dominant effect of known *Su(var)s* were carried out at 25 °C, 70% humidity on regular cornmeal sucrose-based medium (Shaffer et al., 1994). In each cross, male flies exhibiting a representative eye phenotype for a given reporter line were crossed

to female virgins carrying the specified modifier mutation. The 3X 4^{th} line has one copy of the normal 4^{th} chromosome and one copy of the attached 4^{th} chromosome. More detailed information on modifier lines used is listed in Supplemental Table 1. Standard balancers are used to maintain the mutation in each stock.

Inverse PCR and sequencing

Inverse PCR to amplify the region flanking the insertion site was done as previously described (Sun et al., 2004). The PRC product was than treated with ExoSAP (Affymetrix) and sequenced using BigDye Terminator v1.1 (Applied Biosystems) following vendor's instructions. The sequence results were then analyzed using NCBI BLAST with the nr database.

In situ hybridization

In situ hybridization on metaphase chromosomes from third instar larval neuroblasts was done as previously described (Dimitri, 2004). The probe used in this study was the P element reporter containing *hsp26-pt* and an *hsp70*-driven *white* gene (Wallrath and Elgin, 1995).

PEV assay

Ethanol based pigment extraction and quantification was essentially done as previously described (Sun et al., 2004) with some minor adjustments. The overnight incubation step at 4°C was omitted. To increase the throughput and consistency, a Mixer Mill Mm 300 was utilized to homogenize the sample and a plate reader was used for spectroscopy. For each genotype, three to five samples were measured for pigment level; each sample is composed of five male flies (3~5 days old) randomly selected form the population.

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Figure Legend

Figure 1. Sequencing and *in situ* hybridization mapping locate the Y-linked PEV reporters in the telomeric transposon arrays of Ys. (a) Alignment of reporter insertion flanking sequences to a consensus sequence of telomeric retro-transposon TART-B2. The 1 Kb region in the 3'-UTR harboring the insertion sites is magnified. The effective sequence read length for each reporter line is represented by the red line aligned to the region. The red dot at the end of each

red line indicates the 5' end of the reporter. The reporter flanking sequence of line 5m298 starts in the 3' end of a TART-B2 element and extends upstream to a neighboring HeT-A element, suggesting insertion into a partial fragment of TART. (b) *In situ* hybridization images of the metaphase chromosomes from third instar larval brain squashes. Only the Y chromosome of the representative metaphase spread is shown. DAPI staining is pseudo-colored in blue and the hybridization signal is in red. (C: centromere, Y^L: long arm, Y^S: short arm)

Figure 2. Response of Y-linked PEV reporter lines to mutations in well-known modifiers of PEV and TPE (dominant effects). (a) Pigment level quantification representing the level of PEV. Progeny from a cross with yw^{67c23} is used as the wild type control. The allele used in each cross is shown on the X-axis. (Bars represent the average pigment level ± standard error.) (b) Representative pictures showing the dominant impact of the mutations on the fly eyes. The allele used for each modifier is listed below each column. The reporter line used is shown to the left of each row.

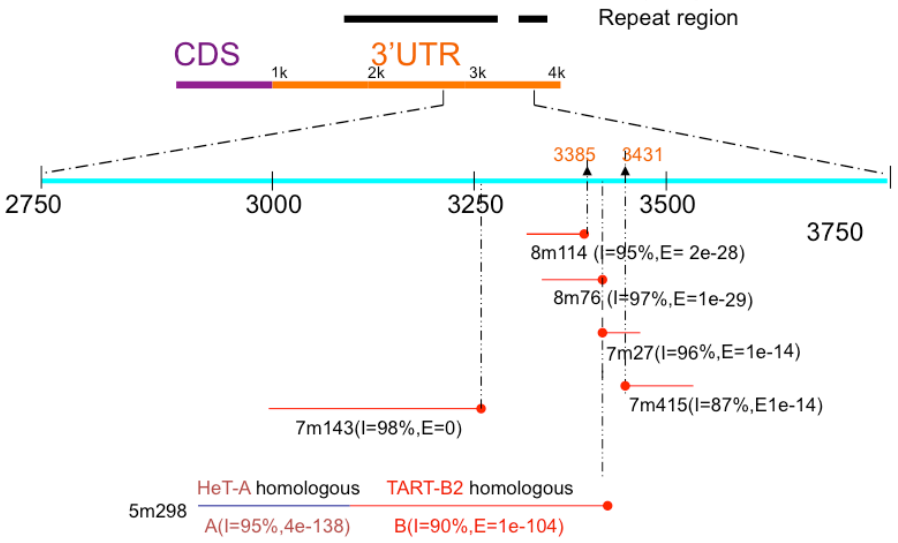
Figure 3. Impact of mutations in HMTs and of dosage of the 4^{th} chromosome on the level of variegation of the Y-linked reporters. (a) Pigment level quantification showing the level of expression. 1X 4^{th} and 3X 4^{th} represent the copy number of the 4^{th} chromosome in the assayed flies. Note that the control line yw^{67C23} has 2X 4^{th}. (See Supplemental Table 1 for information on the fly lines used.) (b)

Representative pictures showing the dominant impacts on PEV in the fly eye from mutations disrupting HMT activities.

Figure 4. Impacts of mutations in components of the small RNA pathways on the level of variegation of the Y-linked reporters. (a) Pigment level quantification indicating the extent of the suppression of PEV. Pathways requiring the genes tested are indicated below the allele names. (b) Representative pictures showing the dominant impacts on PEV in the fly eye from mutations disrupting small RNA pathways.

Figure 5. An ectopic *1360* element enhances Ys telomeric PEV via a piRNA-dependent mechanism. (a) Diagram showing the construct used in this line. FRT sites (gray triangles) flanking the ectopic copy of the *1360* element allow a FLPase mediated excision of the element. (b) *In situ* hybridization image of metaphase chromosomes of line 1250. The DAPI staining is pseudo-colored in blue and the hybridization signal in red. (c) Pigment level quantification comparing the impact on reporter expression of mutations in different small RNA pathway components with and without the ectopic copy of the *1360* element in the reporter. (d) Representative pictures comparing dominant impacts on PEV in the fly eye from mutations disrupting small RNA pathways, with (+) and without (-) the ectopic copy of the*1360* element in the reporter.

Fig 1a



TART-B2 transposon **(DMU14102)**

Fig 1b

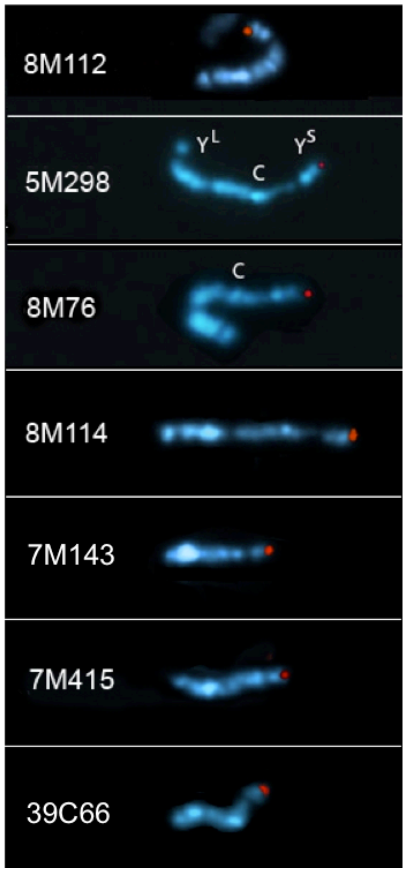
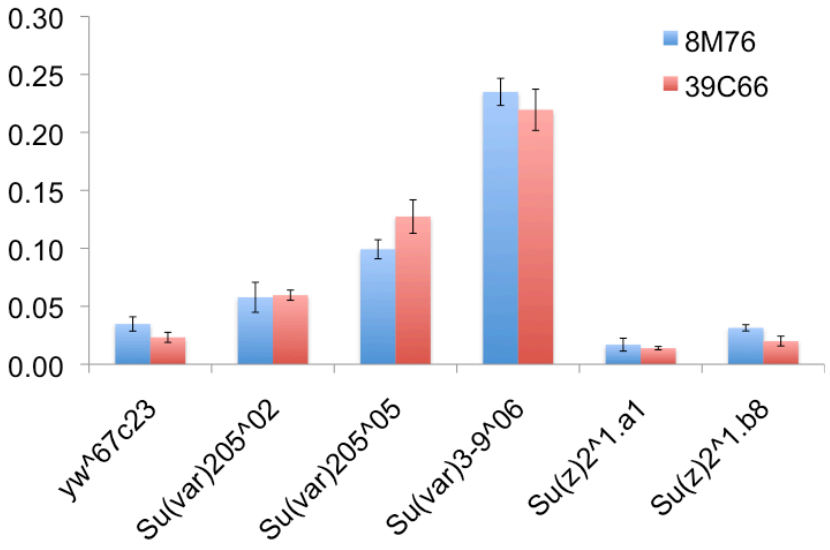


Fig 2a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yw^67c23
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Fig 2b

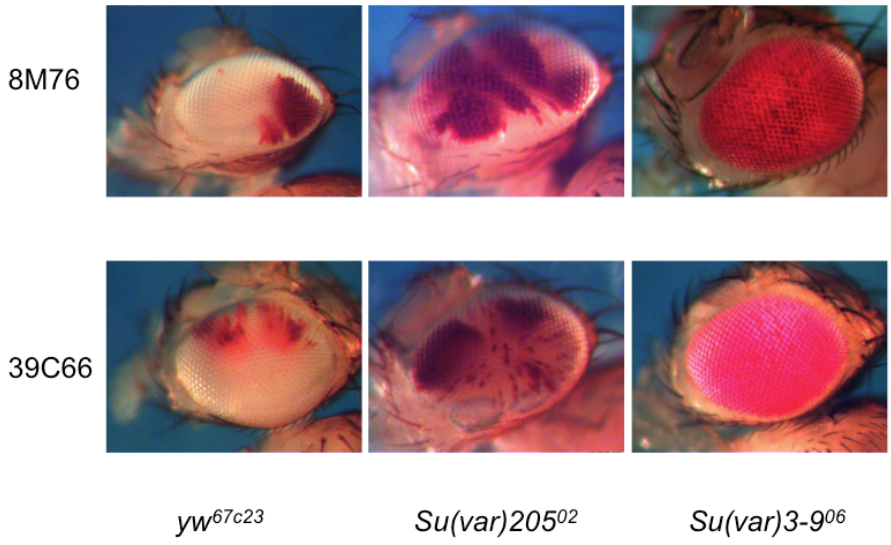
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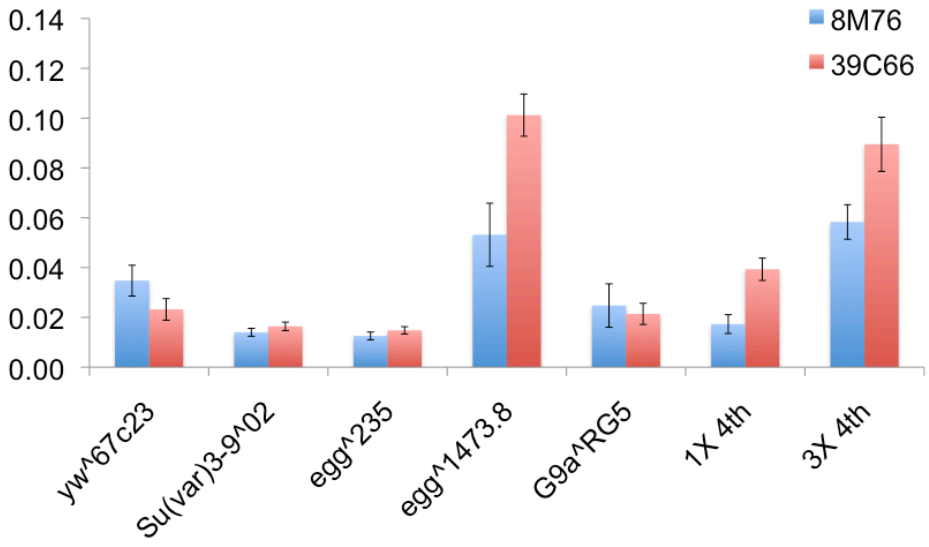
Fig3a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G9a^RG5
1X 4th
3X 4th

Fig3b

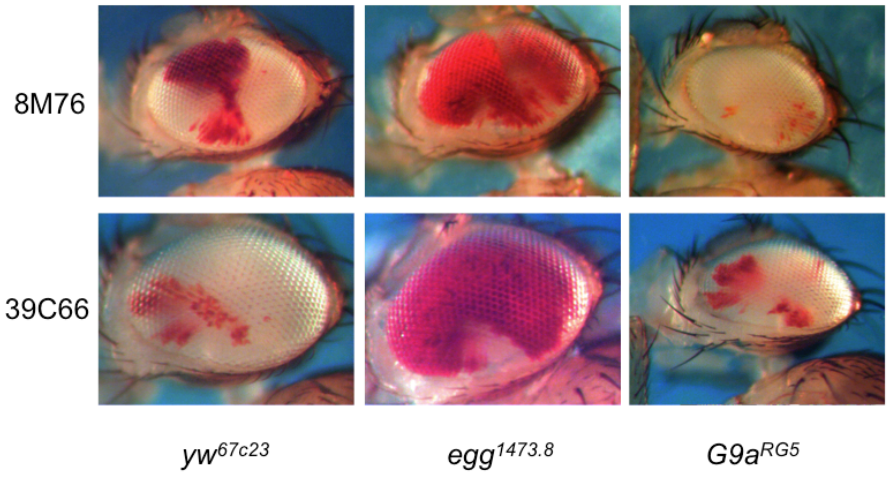
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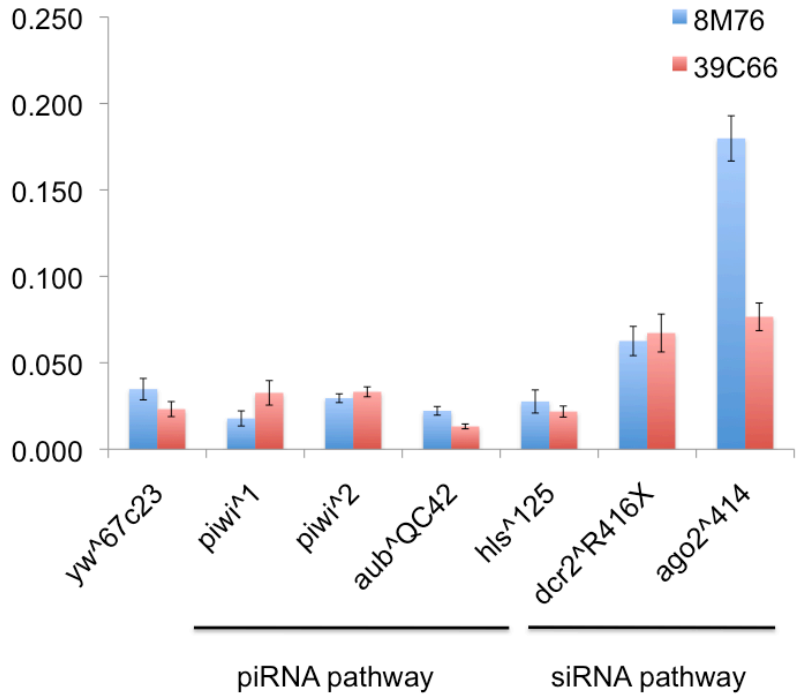
Fig4a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ago2^414
piRNA pathway
siRNA pathway

Fig4b

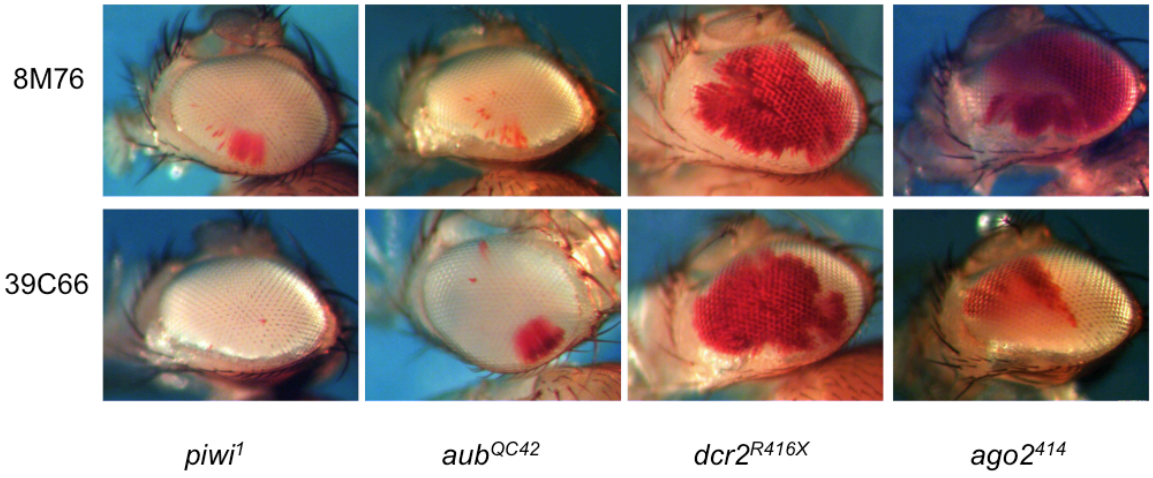
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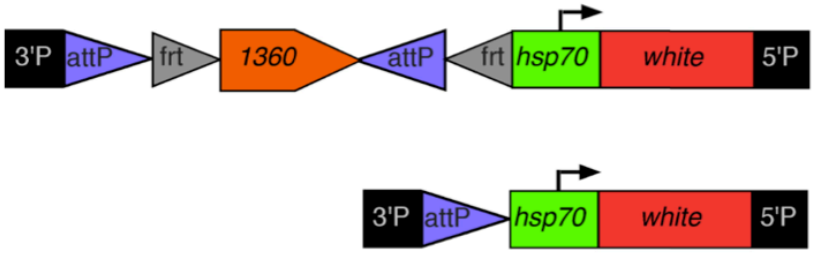
Fig5a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attP
hsp70
white
5'P

Fig5b

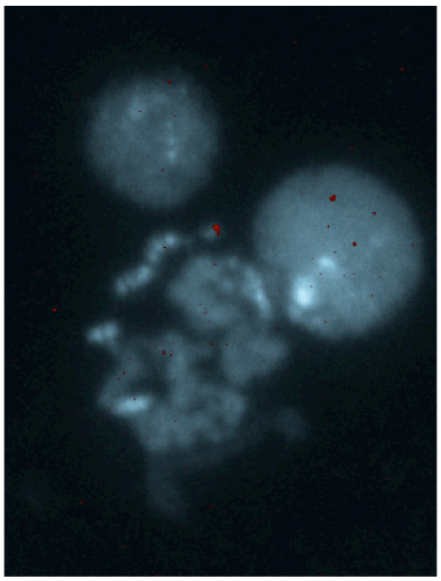
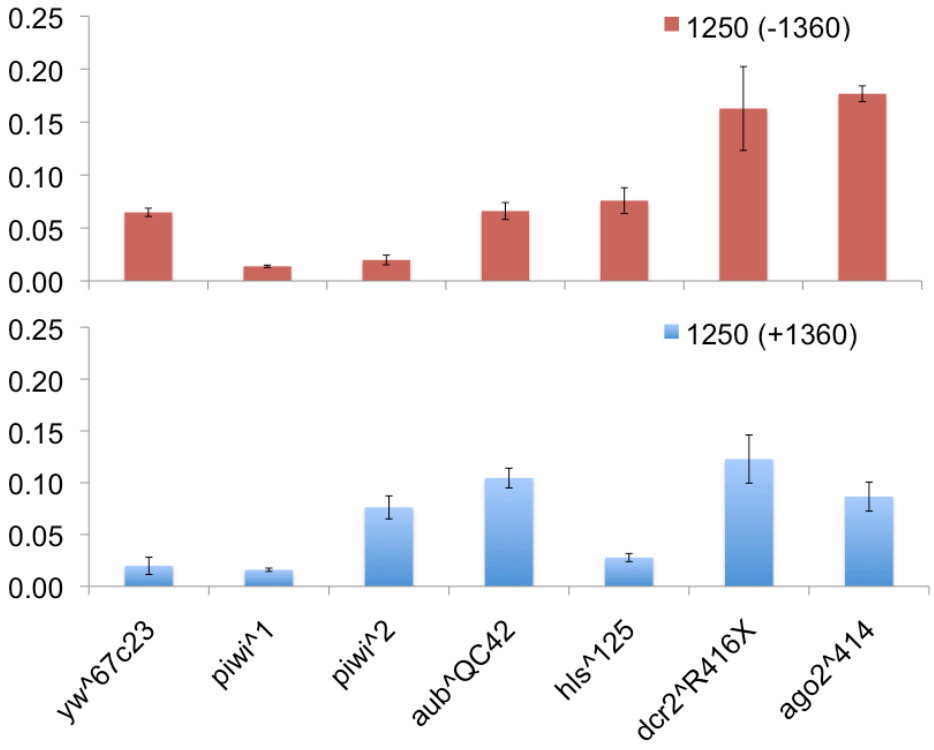


Fig5c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0.20
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0.10
0.05
0.00
1250 (+1360)
0.25
0.20
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0.10
0.05
0.00
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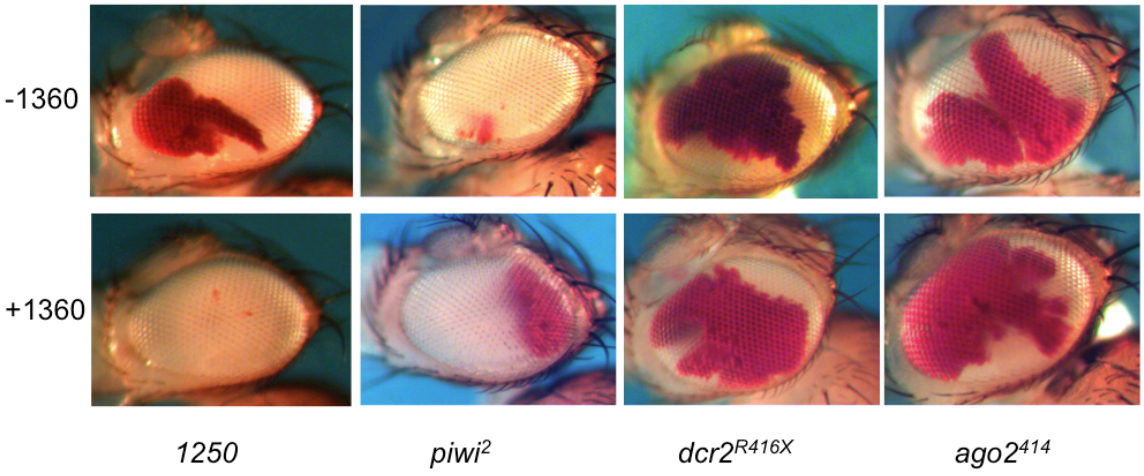
piwi^1
piwi^2
aub^QC42
hls^125
dcr2^R416X
ago2^414

Fig5d


-1360
+1360
1250
piwi2
dcr2R416X
ago2414

Supplement Table 1 List of fly lines used in this study

Stock NO.	Genotype	Source
NA	*ago2^{414}*	Siomi lab
BL4968	*w^{1118}; aub^{QC42} cn^{1} bw^{1}/CyO*	Bloomington Stock Center
BL1785	*C(4)RM, ci[1] ey[R]/0*	Bloomington Stock Center
NA	*yw; dcr2^{R416X} / CyO*	Carthew lab
BL30569	*w*; bw^{1} egg^{1473}/SM1*	Bloomington Stock Center
BL30566	*w*; bw^{1} egg^{235}/SM1*	Bloomington Stock Center
NA	*G9a^{RG5}*	Spierer lab
NA	*hls$^{\Delta 125}$*	Birchler lab
NA	*yw; Su(var)205^{02} / CyO*	Lab stock
NA	*yw; Su(var)205^{05} / CyO*	Lab stock
NA	*yw; Su(var)3-9^{02} / TM3-sb*	Reuter lab
NA	*w; Su(var)3-9^{06} / TM3-sb*	Reuter lab
BL5549	*Su(z)2$^{1.a1}$/CyO*	Bloomington Stock Center
NA	*Su(z)2$^{1.b8}$/CyO*	Lab stock
NA	*w; piwi^{1}/ CyO*	Lin lab
NA	*w; piwi^{2}/ CyO*	Lin lab
NA	*yw^{67c23}*	Lab stock

Chapter 4

Drosophila Piwi functions downstream of piRNA production mediating a chromatin-based transposon silencing mechanism in female germ line

Drosophila Piwi functions downstream of piRNA production mediating a chromatin-based transposon silencing mechanism in female germline

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Running Head: Germline Piwi in transposon silencing

Key Words: Piwi, HP1a, transposon, heterochromatin, piRNA

Classification: Biological science, Genetics

Abstract

Transposon control is a critical process during reproduction. The PIWI family proteins can play a key role, using a piRNA-mediated slicing mechanism to suppress transposon activity post-transcriptionally. In *Drosophila melanogaster*, Piwi is predominantly localized in the nucleus, and has been implicated in heterochromatin formation. Here we use female germline-specific depletion to study Piwi function. This depletion of Piwi leads to infertility and to axis specification defects in the developing egg chambers; correspondingly, widespread loss of transposon silencing is observed. Germline Piwi does not appear to be required for piRNA production. Instead, Piwi requires Aubergine (and presumably secondary piRNA) for proper localization. A subset of transposons that show significant over-expression in germline Piwi-depleted ovaries exhibit a corresponding loss of HP1a and H3K9me2. Germline HP1a depletion also leads to a loss of transposon silencing, demonstrating the functional requirement for HP1a enrichment at these loci. Considering our results and those of others, we infer that germline Piwi functions downstream of piRNA production to promote silencing of some transposons via recruitment of HP1a. Thus in addition to its better-known function in post-transcriptional silencing, piRNA also appears to function in a targeting mechanism for heterochromatin formation mediated by Piwi.

Introduction

Transposons are molecular parasites known to play critical roles in the biology of their host in multiple ways, including being a major force shaping the evolutionary history of a lineage (1, 2). For individuals, germline defense against transposon invasion and mobilization is necessary to maintain the fidelity of genome transmission and general fitness of the offspring. Several different systems involving small RNAs have evolved in eukaryotes for transposon control (3). In many cases, a system involving an RNA-Dependant-RNA-Polymerase (RDRP) amplification and Dicer processing of precursor transcripts is utilized (4, 5). This endogenous small RNA defense mechanism shares many features with the RNAi mechanism first described in *Caenorhabditis elegans* (6). However, in some animals a distinct small RNA defense mechanism has been described (7) utilizing small RNAs that interact specifically with the PIWI clade of argonaute proteins (piRNA) (8-12). Production of piRNA is independent of Dicer enzymes (11), and correspondingly these small RNAs are slightly larger in size (24~30 nts). Rather than use of an RDRP, the amplification of piRNAs has been reported to rely on reciprocal slicing of single-stranded precursor transcripts by PIWI proteins, a process referred to as Ping-Pong amplification (8, 9).

In the *Drosophila melanogaster* female gonad, two distinct piRNA pathways have been identified that drive transposon silencing in the germline and the soma, respectively (13-15). In the germline, piRNA biogenesis involves both primary processing and a secondary amplification pathway (ping-pong amplification) (8, 9, 16), while piRNAs in the soma are generated solely from

primary transcripts (primary pathway) (14, 15, 17).

Despite recent progress, the mechanisms utilized by piRNA to promote silencing are not clear; evidence supporting both transcriptional and post-transcriptional silencing mechanisms has been reported (10, 18-21). The fly genome codes for three PIWI family argonaute proteins used in the piRNA pathways – Piwi, Aub and AGO3 (22). While Aub and AGO3 are restricted to the germline cytoplasm, Piwi localizes predominantly in the nucleus, while still present in the cytoplasm of both the germline and the ovarian soma (8, 10, 23). Correspondingly, Piwi appears to be a key component of both germline and somatic piRNA pathways (14, 15). Aub and AGO3 are the enzymes that generate the 5' end of secondary piRNAs (8, 9). However, the exact role(s) of Piwi, potentially distinct in germline and soma, remains to be elucidated.

Piwi was originally identified as a gene required for maintenance of germline stem cells in *D. melanogaster* (24). Its identification as an argonaute protein (22) led to the identification of piRNAs and their role in transposon silencing. In Drosophila, Piwi was first proposed to take part in the "ping-pong" amplification of secondary piRNAs, which drives a robust post-transcriptional transposon silencing mechanism (8, 9). However, recent high-throughput sequencing analysis (14) has revealed that Piwi is not required for ping-pong amplification; nonetheless, a role for Piwi in germline transposon silencing has been demonstrated (25). How germline Piwi functions in transposon silencing is thus an open question.

While Piwi is likely not involved in the cytoplasmic ping-ping amplification,

it could participate in other steps of piRNA biogenesis. Alternatively, Piwi could function directly in transposon silencing by utilizing piRNAs. The majority of Drosophila piRNAs map to the pericentric or telomeric heterochromatin (8, 10). In *S. pombe* the RITS complex utilizes both an argonaute protein, Ago1, and an HP1 protein, Chp1, in targeting heterochromatin assembly (26). Drosophila Piwi and HP1a interact directly in the yeast two-hybrid system and co-immunoprecipitate from embryo lysates (18). *In vitro* studies indicate that the Piwi N-terminal peptide binds to a dimer of the HP1a chromo shadow domain using a PXVXL motif (27). These observations suggest a role for Piwi in targeting HP1a to silence transposons through a chromatin-based mechanism. However, Piwi is capable of slicing an RNA substrate *in vitro* (10), which argues for a post-transcriptional or co-transcriptional silencing function.

Most prior functional analyses of Piwi have used mutant lines deficient in Piwi in both germline and soma (11, 12, 14). This results in a mixture of germinal and somatic *piwi* phenotypes, and could reflect the mixed features of Piwi in two (or more) independent pathways. Further, a lack of functional Piwi in the ovarian soma leads to a block in oogenesis, with pleiotropic consequences (23).

In this study, we specifically deplete Piwi in the germline to gain a mechanistic understanding of its function there. We observe that germline Piwi apparently functions downstream of piRNA production to silence a subset of transposons; loss of transposon silencing generally correlates with loss of HP1a and H3K9me2 from the repetitious element. The results support a chromatin-based transcriptional silencing mechanism dependent on germline Piwi, and

suggest a possible mechanism for targeting heterochromatin formation.

Results

We depleted Piwi using a female germline-specific GAL4 driver, NGT40 (28), driving an RNAi knockdown construct (29) in conjunction with over-expression of DCR2 (Suppt Fig. 1). To ensure target specificity, two RNAi knockdown hairpins with no overlapping 19-mers (examined by a sliding window analysis) were used (see Materials and Methods). Both hairpin constructs have Piwi as their only target in the fly genome. Knockdown experiments using these hairpins result in a decrease in the level of *piwi* transcript in the ovaries to one third that of the wild type (Fig. 1A). Since the transcript level was measured using whole ovaries, the majority of the residual *piwi* transcripts likely come from the somatic follicle cells. Immunofluorescent staining of the knockdown ovaries with Piwi antibody shows that the signal in the germ cells is strongly depleted while the signal in the surrounding somatic follicle cells is not affected (Fig. 1B), demonstrating that knockdown is significant and specific to the germline.

As reported earlier using a mitotic recombination strategy (23), germline Piwi knockdown does not block oogenesis. However, the eggs laid show a high frequency of collapse and a very low rate of hatching (Suppt Table 1). A significant portion of the embryos produced here from germline Piwi knockdown females show fused or absent dorsal appendages (Fig. 1C). Correspondingly, Gurken localization to the dorsal region of the developing oocyte (required for specification of the dorsal/ventral axis) is decreased (Fig. 1D). A similar shift in Gurken localization and a concomitant dorsal appendage defect have been

observed with other mutations in the piRNA pathway (30).

It was previously suggested that the axis polarity specification defects resulting from mutations in piRNA pathway genes are likely a secondary effect due to loss of transposon control (30, 31). Transposon transposition creates DNA double strand breaks; a DNA damage response can occur, leading to a checkpoint arrest and polarity specification defects. Indeed, Kalmykova *et al*. have shown that the progeny of *piwi* mutants can exhibit new insertion sites for the *mdg1* transposon (32). This report of actual transposition events provides strong evidence linking transposon activity with the polarity specification defects commonly observed in mutants deficient in piRNA pathway components, and seen here.

Examining transposon expression levels in these Piwi germline knockdown lines, we observe a loss of silencing for over half of the ca. 30 transposons tested by quantitative PCR using total ovarian cDNA (Table 1). Telomeric retrotransposon HeT-A and LTR retrotransposon Burdock show the most dramatic effects. In general, transposons that show increased expression in germline Piwi knockdown lines were the same as those that showed increased expression in an *ago3* mutant line as reported in Li et al (13) (Table 1). This argues that germline Piwi functions in the same pathway as Aub and AGO3 (8, 9). However, three cases clearly do not follow this pattern: transposons Max, Idefix and Invader1 are significantly up-regulated in Piwi germline-knockdown ovaries (Table 1), but are reported to show little to no response to an *ago3* mutation (13). This discrepancy suggests an additional role for Piwi.

To confirm that the observed transposon over-expression phenotype is a direct consequence of Piwi depletion in the germline, we used a DFS-FLP strategy to populate the entire germline with homozygous *piwi*^{1} germ cells (Suppt Fig. 2) (33), and assayed the effect on transposon expression with and without the presence of a wild type Piwi rescue construct. Germline *piwi* flip-out ovaries exhibit strong up-regulation of expression from transposons HeT-A, Burdock, Blood and Invader1 (Suppt Fig. 3), similar to that seen for germline Piwi knockdown ovaries (Table 1). A wild type Piwi transgene results in rescue, with all four tested transposons reverting to wild type levels of expression (Suppt Fig. 3). This result confirms the specificity of the knockdown effect and indicates that some transposons, e.g. Invader1, require Piwi but not AGO3 (and the secondary piRNA it helps produce) for proper regulation, suggesting an additional mechanism. In addition, in the cases tested, a Piwi transgene with a valine to alanine substitution at amino acid 30, Piwi^{V30A} (18), also rescues the over-expression phenotype (Suppt Fig. 3), suggesting that an intact PXVXL motif is not required for Piwi to silence transposons in the germline.

Using the DFS-FLP strategy to replace wild type *piwi* with the *piwi*^{1} loss-of-function allele (Suppt Fig. 2), we next looked at the impact of germline Piwi depletion on the localization pattern of Aub. Mutations disrupting the ping-pong amplification process can lead to mis-localization of Aub from the peri-nuclear structure *nuage* (13, 34), the proposed site of secondary piRNA production (34). Similar to earlier observations using mutants that disrupt Piwi in both germline and soma (14), we found no impact on Aub localization to the *nuage* (Fig. 2A).

The lack of change is in agreement with the earlier finding that germline Piwi is not required for the ping-pong amplification process (14).

To ask whether germline Piwi is involved in other steps of the piRNA biogenesis pathway, we assayed piRNAs originating from three independent loci, HeT-A (a telomeric non-LTR retrotransposon), Roo (an abundant LTR retrotransposon) and AT-chX-1 (a non-transposon repetitive DNA element) (35), by Northern blot. In contrast to findings using mutants that disrupt *piwi* in both germline and soma (11), we did not observe a disruption of piRNA production in germline *piwi*^{1} mutant ovaries. Instead, we see an increase in piRNA from all three elements (Fig. 2B), suggesting that germline Piwi is not required for piRNA biogenesis. Results from germline Piwi knockdowns are similar (Suppt Fig. 4).

These results indicate a role for germline Piwi downstream of piRNA production, potentially downstream of Aub/Ago3 activity. Germline-specific knockdown of Aub (Fig. 3A) results in a strong depletion of the Roo element, AT-chX-1, and 3'-UTR HeT-A piRNAs in ovaries (Suppt Fig. 4a, 4b). All of these probes hybridize extensively with ping-pong amplified piRNAs (Suppt Table 2); their depletion confirms that germline Aub knockdown disrupts the ping-pong amplification cycle. Inspection of transposon expression levels shows significant up-regulation of multiple transposons on Aub knockdown (Fig. 3B). In particular, retrotransposons HeT-A and Burdock, which showed strong up-regulation upon germline Piwi depletion (Table 1, Suppt Fig. 3), show similar up-regulation here, supporting the idea that Piwi and Aub are functioning in the same pathway.

No significant impact on Piwi expression levels is observed in Aub

knockdown ovaries (Fig. 3A). However, mutations in piRNA pathway components can lead to mis-localization of Piwi protein (14, 36); aub^{QC42}/aub^{HN2} results in a strong decrease in the level of Piwi in the nucleus (13). Here immuno-fluorescent staining experiments show a notable depletion of Piwi signal in the germline nuclei of Aub knockdown ovaries (Fig 3C(b)). Since the total Piwi protein level is not affected (Fig. 3A), this suggests that Aub knockdown results in a dispersed Piwi localization pattern. The diffuse nuclear localization is most obvious in early stage egg chambers (Fig. 3D), while the reduced nuclear-to-cytoplasmic Piwi signal ratio is more obvious in latter stage egg chambers (Fig. 3E). This Piwi staining pattern in early stage egg chambers (Fig.3D) is very similar to that reported in *zucchini* mutant ovaries (36). The evidence as a whole argues for a role for Aub in Piwi nuclear localization, and indicates a function for germline Piwi in transposon silencing downstream of piRNA production.

Previous studies have supported a role for the piRNA pathway in heterochromatin-dependent silencing (18, 20, 21), in particular implicating a direct interaction between Piwi and HP1a (18, 27). Chromatin immunoprecipitation experiments show a significant loss of HP1a following germline Piwi knockdown at five transposon sites out of seven tested, looking at their promoter region or 5' end (Fig 4A). The Roo element is not regulated by germline Piwi (Table 1), and we observe little to no impact on it's HP1a enrichment (Fig 4A). Amongst the transposons tested, HeT-A and Burdock show the most dramatic depletion of HP1a, while Blood, Bari1 and Invader1 also show a significant decrease (Fig 4A). Similar (but less potent in the case for HeT-A)

results were observed when the internal regions of these transposons were examined (Suppt Fig. 5). Transposon Jockey shows little to no HP1a depletion, suggesting additional mechanisms for Piwi silencing.

To ask whether this Piwi-dependent enrichment of HP1a at transposon sites is established through a mechanism downstream of secondary piRNA production, we examined the impact of Aub knockdown on HP1a enrichment. Transposons HeT-A, Blood and Burdock all show loss of HP1a enrichment similar to that seen in germline Piwi knockdown (Suppt Fig 6). A similar lack of impact on the Roo element is also observed. These findings suggest that for transposons HeT-A, Blood and Burdock, Piwi recruits HP1a to transposon sites through a mechanism downstream of secondary piRNA production.

For six of seven transposons tested in germline Piwi knockdown ovaries, we observe a strong correlation between depletion of HP1a occupancy and increase in transcript levels (Fig 4A, Table 1). To directly test this relationship, we examined transposon expression levels in germline HP1a-depleted ovaries. Germline HP1a knockdown blocks oogenesis and results in abnormal ovaries (Suppt Fig. 7), but the incomplete penetrance and variable expressivity of this phenotype in our system allowed us to prepare ovarian cDNA (primed with random hexamers) to profile expression of these transposons. We observe a high degree of correlation between the two data sets (Fig 4A, 4B). Significant up regulation of expression in the absence of germline HP1a (Fig 4B) is seen for all five transposons that show significant HP1a depletion at their 5' end/promoter region in germline Piwi knockdown ovaries (Fig 4A). Transposons Jockey and

Roo show no significant change in expression (Fig 4B), correlating with the lack of impact on HP1a enrichment levels in germline Piwi knockdown ovaries (Fig 4A). These results demonstrate that enrichment of HP1a is critical for maintaining proper control of expression for a subset of transposons.

HP1a functions as a structural component of pericentric heterochromatin (37), binding di- and tri-methylated histone H3 lysine 9 (H3K9me2/3) through its chromo domain (38, 39) and interacting with SU(VAR)3-9, a histone 3 lysine 9 methyltransferase (40). To look for evidence of a similar mechanism here, we examined the impact of germline Piwi knockdown on the enrichment level of H3K9me2 at those transposons. Strong reductions in H3K9me2 levels are seen at the HeT-A promoter region and at the 5' end of Burdock, with significant but less potent depletion at the 5' ends of Blood and Bari1 (Fig 4C). Taken together with the observed loss of HP1a occupancy at these same sites (Fig 4A), the results suggest that the Piwi dependent, HP1a dependent germline transposon silencing is likely functioning through such heterochromatin formation.

Discussion

The results above lead us to conclude that germline Piwi functions in silencing a subset of transposons either through recruiting HP1a to the transposon sites, likely directed by piRNAs, or through an unknown mechanism(s) to maintain HP1a enrichment at transposon sites. The former interpretation is supported by the ChIP results obtained from Aub knockdown ovaries (Suppt Fig 6) and fits well with the small RNA targeting model for heterochromatin formation first described in fission yeast, *Schizosaccharomyces*

pombe (26, 41). However, as in many previous studies, we find that not all transposable elements behave in the same way, and that it is necessary to invoke more than one mechanism to achieve silencing of all transposons. For example, we observed three cases that exhibit obvious up-regulation in germline Piwi knockdown ovaries that did not respond to mutations in *ago3* (Table 1). One possibility is that Piwi functions in these cases through a primary piRNA mediated mechanism (16).

We find that germline Piwi is not required to maintain wild type levels of piRNA (Fig 2), which is in contrast to an earlier study (11) describing a significant decrease in Roo piRNA levels in *piwi* homozygous mutant ovaries. The major difference between the two studies likely comes from the difference in tissue type. Depletion of Piwi specifically in the germline, as done here, allows oogenesis to occur normally (23), whereas depletion in the ovarian soma as well leads to blockage of oogenesis, resulting in ovariole bundles composed mostly of somatic cells (24). Thus the signals detected in the latter experiments probably reflect functions of somatic Piwi. Using an Ovarian Somatic Cell line (OSC), Saito et al have shown that Piwi is required to maintain normal piRNA levels in these cells (17). While the mechanism remains unclear, their results in soma taken together with our observations in germline highlight a distinction in Piwi function between the two tissues.

The significance of the observed increase for HeT-A and AT-chX-1 piRNA levels in germline Piwi depleted ovaries (Fig 2B) remains unclear. One attractive interpretation would be that the increase in transcript levels in the absence of

germline Piwi could result in an increase in substrate available for the ping-pong amplification cycle (8, 9). The strong depletion in AT-chX-1 and Roo piRNA levels in Piwi-Aub double knockdown ovaries (Suppt Fig 4a) supports this idea.

In Aub knockdown germline, we observe a more diffuse Piwi localization pattern (Fig 3). One attractive interpretation would be that germline Piwi requires loading of piRNAs to be licensed for its nuclear entrance (13). Depletion of Aub leads to disruption of the ping-pong amplification cycle and would therefore disrupt any nuclear localization of Piwi dependent on secondary piRNA interaction. The remaining nuclear Piwi signal could come from Piwi proteins loaded with primary piRNAs, or alternatively result from incomplete Aub depletion in the knockdown ovaries.

Jockey appears to be a singular case amongst transposons tested here. While it requires both Piwi and AGO3 for proper control of expression (Table 1), it does not seem to respond to germline Aub knockdown (Fig 3B). Moreover, the chromatin immunoprecipitation results and HP1a knockdown results indicate that Jockey expression is regulated via a mechanism that is independent of HP1a. As Aub knockdown leads to an increase in cytoplasmic Piwi relative to the nuclear fraction (Fig 3E), Piwi could execute Jockey silencing in the cytoplasm. Further studies on how germline Piwi silences Jockey could be very informative in understanding how Piwi functions in general.

Although our results clearly indicate that germline Piwi functions through recruiting HP1a to some transposon sites to induce local heterochromatin formation and silence transposons, the actual mechanism of HP1a recruitment

by Piwi remains to be determined. The direct interaction between Piwi and HP1a using a PXVXL motif, observed both *in vitro* (27) and in a yeast two-hybrid assay (18) provides a possible means to mediate this process. However, a direct test using a V30A mutant *piwi* transgene showed rescue of transposon silencing in the *piwi*^{1} germline, indicating that germline Piwi does not require an intact PXVXL motif for this silencing function (Suppt Fig. 3). Nonetheless, the results above showing loss of HP1a deposition in response to Piwi depletion, taken together with the previously reported co-immunoprecipitation of Piwi and HP1a (18), argue for a link; we suggest that there are likely additional interactions bridging between Piwi and HP1a. Alternatively, Piwi could recruit HP1a through an indirect mechanism, yet to be elucidated. Further exploration will be needed to determine the mechanisms for Piwi dependent recruitment of HP1a.

In summary, our results using a system that can deplete Piwi specifically in the female germline provide novel findings that correspond well with the current literature and support the hypothesis of a chromatin-based transposon silencing mechanism for germline Piwi in Drosophila (see model, Suppl Fig 8). Our observations are in agreement with an earlier study from Gvozdev and colleagues, who used *spn-E* mutants to look at the impact of piRNA pathway mutations on chromatin structure at transposon sites (20). In addition, our study further positions Piwi downstream of piRNA production to function in directing assembly of a proper chromatin structure at transposon sites to achieve silencing.

Materials and Methods

Fly stocks, husbandry and genetics

All crosses were performed at 25 ºC, 70% humidity using regular cornmeal sucrose-based medium. Full genotypes of the fly lines used are listed in Supplement Table 3. For female germline knockdown experiments, male flies from the driver line were crossed with female virgins from the respective hairpin target lines (Supplement Fig. 1). Hairpin lines used in this study were *yw;+/+;P{my*$^{+}$*=UAS-PIWIhp*^{8}*}* (piwiKD2), *w*^{1118}*;P{GD11827}22235* (piwiKD1), *w*^{1118}*;P{GD12524}v31995* (HP1aKD), and *w*^{1118}*; P{GD11831}v30125* (aubKD) (29) (abbreviations given in Supplement Table 5). The DFS-FLP experiment was carried out as previously described (23) (Supplement Figure 2). Ovaries were dissected from 3 to 5-day-old females provided with fresh yeast overnight.

Hairpin transgenic line construction

Hairpin line construction was carried out as previously described (42) except that the *piwi* fragment was amplified from a cDNA clone, GM05853 (43), using the following primer pair: forward 5'-GCT CTA GAT CCG GTT GAG CTG GTA TCC AAG AA-3' and reverse 5'-GCT CTA GAA GAT CGT CTC GGT GCG CAT AAC TT-3'. Seven transgenic lines with different insertion sites were recovered (Supplement Table 4).

Immunostaining and confocal imaging

Flies were dissected in EBR (an iso-osmotic buffer) and dissected ovaries were fixed in 6% formaldehyde saturated with heptane (44). Antibodies used for immunostaining are P4D2 anti-Piwi (1:2) (10), 1D12 anti-Gurken (1:20)

(Developmental Studies Hybridoma Bank, University of Iowa), 4D10 anti-Aub (1:200) (35) and C1A9 anti-HP1a (1:10) (45). Phaloidine-Alex568 (Invitrogen) (1:100) was used to stain actin. Secondary antibodies were Alexa Fluor conjugated antibodies from Invitrogen. Images were collected on a Nikon A1 confocol microscope. Each image was averaged over 16 scans of a single focal plane and processed using Image J software and Adobe Photoshop.

Western analysis

Ovarian lysate was prepared as described (46). Electrophoresis was carried out with a 4-20% polyacrylamide gradient gel (Bio-Rad) in SDS running buffer. Proteins were wet-transferred to a 0.45 micron nylon membrane, and the membrane probed with the respective antibodies in 5% milk TBST using the following dilutions: P4D2 anti-Piwi (1:66) (10), 4D10 anti-Aub (1:1000) (35) and 3C7 anti-myosin VI (1:20) (47). HRP (horse radish peroxidase) conjugated secondary antibodies (KPL) and substrates (Millipore) were used according to vendors' instructions to visualize the results.

RT-qPCR and northern blot

RNA was isolated with TRIzol following vendor's instructions. For each biological sample, 20 pairs of ovaries were hand-homogenized in 1ml of TRIzol reagent using a small pestle. RNA templates for RT reactions were treated with DNAse I (Fermantas). cDNA was generated using random hexamers (Invitrogen) and SuperScript III (Invitrogen) following vendor's instructions. Quantitative PCR was performed using iQ SYBR Green Supermix (BioRad) on an ABI 7500 or a Cepheid Smart Cycler. Primers used are listed in Supplement Table 6. Results

were analyzed using the ΔΔCT method (48). Small RNA Northern blots were done as previously described (49) but omitting the size selection step. Probes used are listed in the Supplement Table 7.

Chromatin immunoprecipitation

Chromatin preparation was carried out as previously described (19). Sonication used a Branson sonifier with a microprobe at 100% duty cycle and output setting 2. Four 12 sec bursts with 2 min intervals on ice gave a sample with fragment sizes between 100 to 1000 bp. Immunoprecipitation was carried out following the modENCODE protocol (http://www.modencode.org/Protocols.shtml) using antibodies WA191 (121701) anti-HP1a (50) (1:50), Ab1220 anti-H3K9me2 (lot 765092, Abcam) (1:100) and Ab8580 anti-H3K4me3 (lot 224576, Abcam) (1:100). The relative enrichment of each mark at the designated region was quantified by qPCR. Primers used are listed in Supplement Table 6, lower part. The 5' primers were designed to amplify the junction between LTR and internal TE sequence. For consistent amplicon size, primer pairs are tested by *in silico* PCR on the UCSC genome browser website. The percent input of each IP at each locus was determined using input sample dilutions. Relative enrichment at a given locus was then determined by normalizing the locus percent input over alpha-actinin or 18S ribosomal DNA percent input. The mean of the normalized value from 3 biological replicates is reported.

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Figure Legends

Figure 1. Germline-specific Piwi depletion leads to axis specification defects in developing egg chambers. (A) Quantitative RT-PCR analysis of the *piwi*

expression level in germline Piwi knockdown ovaries. Expression levels are given relative to the RPL32 locus. (Bars represent the mean ± SEM) (B) Piwi antibody staining of developing egg chambers. Piwi depletion specifically in the germline (red arrows) is achieved with either of two independent knockdown constructs without affecting the surrounding somatic follicle cells. (Full genotypes of piwiKD1 and piwiKD2 are given in Supplement Table 5.) (C) The cumulative percentage of dorsal appendage phenotype of embryos produced by germline Piwi knockdown females. (N represents the total number of embryos scored for each genotype.) (D) Gurken (green) immunofluorescent staining of stage 9 developing egg chambers. The oocyte nucleus is indicated (asterisk). DAPI staining (blue) marks the nuclei and the actin filament (red) marks the cell boundaries. Gurken localization is diminished in the Piwi knockdown lines.

Figure 2. Depletion of germline Piwi does not disrupt Aub function. (A) Aub immunofluorescent staining of stage 4/5 egg chambers bearing *piwi*^{1}/+ or *piwi*^{1}/*piwi*^{1} germline. The peri-nuclear structure *nuage* (black arrow) and Aub localization are not perturbed in the *piwi*^{1}/*piwi*^{1} germline. (B) Small RNA northern analysis using three different piRNA probes, HeT-A-2801, AT-chX-1 and Aub-bound roo, along with a microRNA probe, miR-8, as a loading control. (A, B) Genotypes indicated are germline genotype at the *piwi* locus.

Figure 3. Germline Aub knockdown perturbs proper Piwi nuclear localization and leads to over-expression of some transposons. (A) Western analysis of Piwi or

Aub protein levels in Aub knockdown ovaries shows no significant loss of Piwi. Myosin VI is used as the loading control; the volume of lysate loaded in each lane is indicated beneath. (B) Quantitative RT-PCR analysis of transposon expression levels in germline Aub knockdown ovaries. Expression levels are given relative to the RPL32 locus. (Bars represent the mean ± SEM) (C) Piwi immunofluorescent staining of ovarioles. In the Aub knockdown germline, Piwi is barely visible in the nuclei of early stage egg chambers, arrow, in contrast to wild type. (D) The diffuse pattern of Piwi staining in an Aub knockdown germline is most apparent in stage 2/3 egg chambers. (E) Piwi immunofluorescent staining of stage 6/7 egg chambers. DNA staining is shown in red to delineate the nucleus. The overall Piwi signal in the Aub knockdown egg chambers is adjusted so that the signal strength in the germline nuclei matches the corresponding region in the wild type egg chamber. (D, E) Scale bars: 5 μm.

Figure 4. Germline Piwi functions in silencing some transposons through an HP1a dependent chromatin-based mechanism. (A) ChIP-qPCR analysis at 5' ends or promoter regions (as indicated in the label) of a set of transposons using antibodies against HP1a in germline Piwi knockdown ovaries. The enrichment levels are relative to the alpha-actinin locus. (B) qRT-PCR analysis of expression levels for the same set of transposons in germline HP1a knockdown ovaries. Fold expression levels are relative to RPL32 expression. (C) ChIP-qPCR analysis at 5' / promoter regions of a set of transposons using antibodies against H3K9me2 in germline Piwi knockdown ovaries. The enrichment levels are

relative to the 18S ribosomal DNA locus. (A, B and C) Bars represent mean ± SEM of 3 biological replicate experiments.

Table 1. Transposon response to germ-line Piwi knockdown

Element (type) [a]	Fold expression [b]	AGO3 response [c]	AGO3 grouping [d]
Strong [e]			
Bari1 (T)	3.94 ± 1.48	I	I
Blood (L)	4.67 ± 0.03	S	III
Burdock (L)	7.81 ± 0.87	S	I
Diver (L)	4.24 ± 0.09	S	I
HeT-A (N)	8.70 ± 1.44	S	I
Idefix (L)	4.03 ± 0.95	W	III
Invader1 (L)	3.40 ± 1.66	W	II
Invader4 (L)	5.32 ± 1.44	I	I
Max (L)	3.19 ± 0.07	W	I
Intermediate [e]			
1360 (T)	1.91 ± 0.69	I	I
1731 (L)	2.83 ± 1.15	W	I
412 (L)	2.27 ± 0.15	I	III
Accord (L)	1.91 ± 1.60	I	I
Diver2 (L)	2.79 ± 0.87	W	II
Jockey (N)	2.85 ± 0.05	I	I
R1A1 (N)	2.10 ± 0.44	W	I
Rt1a (N)	1.91 ± 0.34	W	I
Weak [e]			
297 (L)	1.21 ± 0.95	W	III
Aurora (L)	1.28 ± 0.01	W	I
Doc (N)	1.56 ± 0.69	W	I
F-element (N)	0.80 ± 0.51	W	I
Gypsy6 (L)	1.43 ± 0.90	W	III
Hopper (T)	1.59 ± 0.53	W	II
INE-1 (S)	1.33 ± 0.98	W	I
Mdg1 (L)	0.76 ± 0.04	W	III
NOF (T)	0.56 ± 0.58	W	I
Opus (L)	0.82 ± 0.52	W	I
Roo (L)	0.28 ± 0.24	W	I
S-element (T)	1.36 ± 0.43	W	I

a. Element type abbreviations used are: T, TIR element; L, LTR retrotransposon; N, non-LTR retrotransposon; S, SINE element.

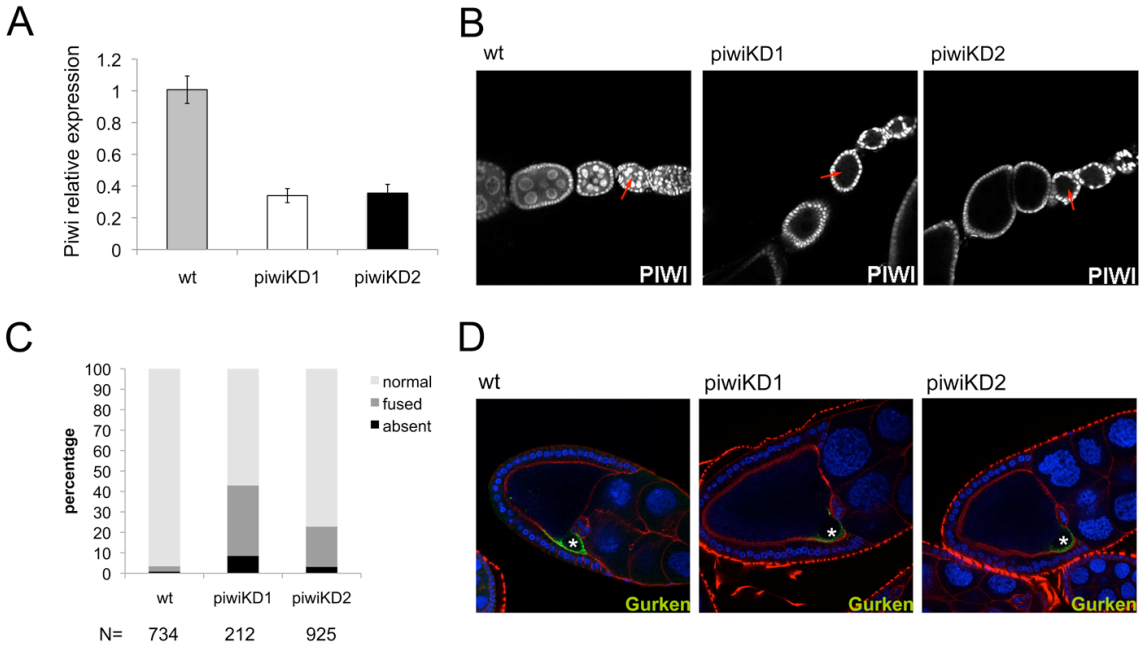
b. Average (±SD) of two Piwi knockdown lines relative to a wild-type control. Data presented using a log 2 scale.

c. Transposon expression in response to an *ago3* mutation. The response is classified based on results from Li et al (13). I, intermediate; S, strong; W, weak

to none.

d. Transposon groupings based on piRNA sequencing results (13).
e. The extent of response is categorized into three groups using cutoff values of 3.0-fold and 1.6-fold increased expression for each transposon (log 2 scale).

Wang_Fig1



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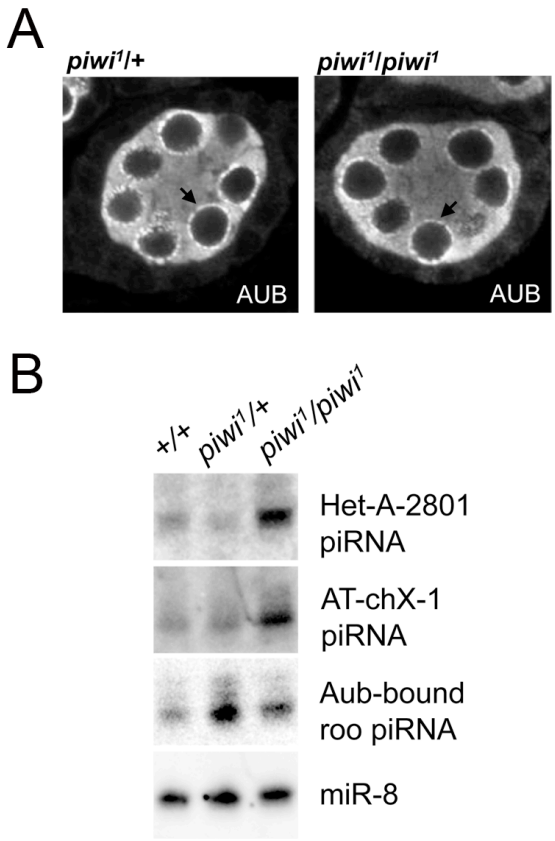

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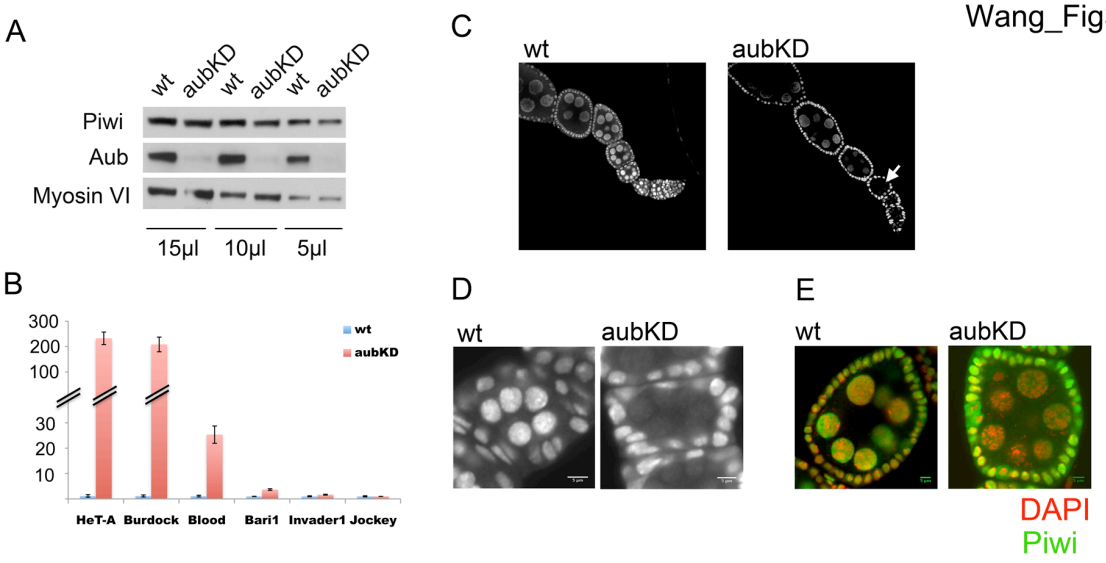

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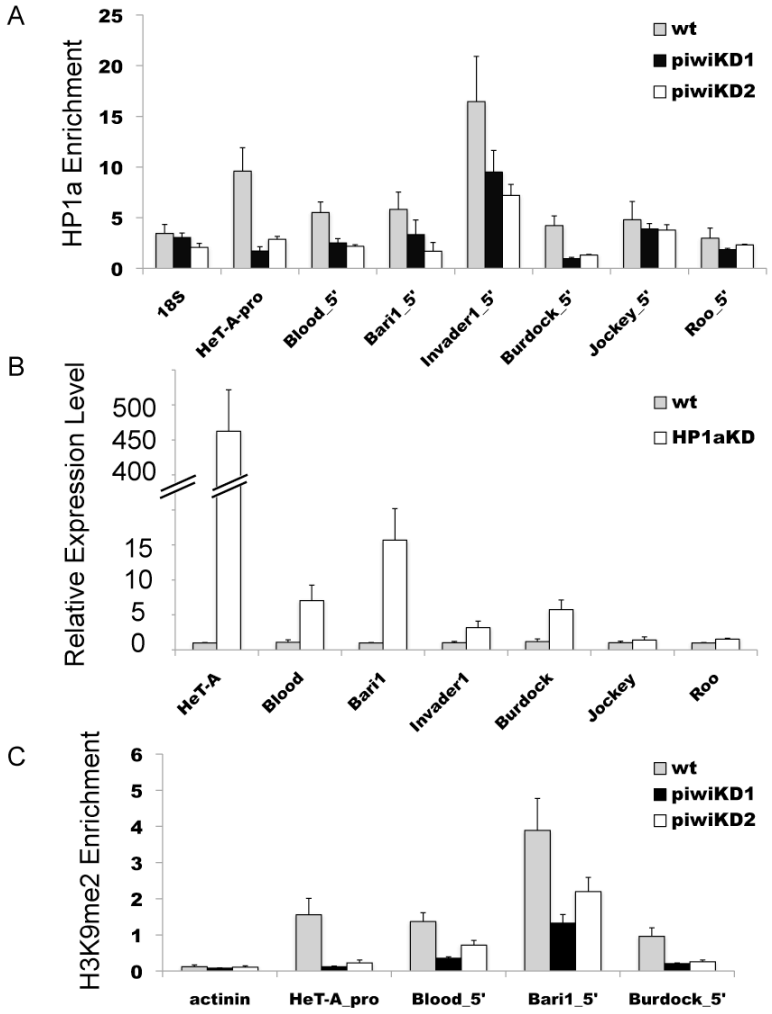


Supplement Figure 1. Schematic representation of strategy for female germline specific Piwi knockdown. A germline specific *nos*-GAL4 driver, NGT40, is used to simultaneously drive germline specific expression of a *piwi* hairpin construct and over-expression of DCR2. The hairpin product is presumably processed by the siRNA pathway and utilized to suppress Piwi translation through a targeted cleavage of *piwi* mRNA. Over-expression of DCR2 in conjunction with the hairpin construct is critical to achieve efficient knockdown in the germline.

Supplement Figure 2. The DFS-FLP strategy generates female flies with a homozygous *piwi*^{1} germline. a, Schematic representation of the DFS-FLP strategy used to generate a *piwi*^{1} germline. Early in oogenesis, heat shock induced flip recombination generates cell populations with 3 different genotypes. After multiple rounds of mitosis through development, all 3 cell populations survive and inter-mingle in the soma. However, the dominant *ovo*^{D} allele blocks the developmental program of germ cells, resulting in a uniform cell population with the *piwi*^{1} genotype in the fully developed germline. **b,** Piwi immunostaining comparing egg chambers with a heterozygous (left) and homozygous (right) *piwi*^{1} germline. In the latter case, one sees a complete lack of staining in the germline nuclei.

Supplement Figure 3. The transposon overexpression phenotype caused by germline Piwi depletion can be rescued by introducing a wild type or a

V30A Piwi transgene. qRT-PCR analysis of transposon expression levels in ovaries of homozygous *piwi*^{1} germline clones in the presence or absence of a *piwi* transgenic rescue construct. Indicated below each column is the germline genotype at the endogenous *piwi* locus and the presence of either a wild type or a V30A *piwi* rescue construct. Bars represent mean ± SEM of three biological replicates. Germline mutant clones were generated via the DFS-FLP strategy (see Supp. Fig 2). Fold expression levels are relative to RPL32 expression.

Supplement Figure 4. Germline Piwi knockdown has little impact on ping-pong amplified piRNA levels, whereas Aub knockdown leads to loss of these small RNAs. a, Small RNA northern analysis using three different piRNA probes, HeT-A-2801, AT-chX-1 and Aub-bound roo to interrogate total ovarian RNA. A microRNA probe, miR-8, is used as a loading control. The genotype abbreviation for each sample is shown by the label on top of the column. (The full genotype can be found in Supplemental Table 5.) The first two columns present data to check the potential impact of DCR2 over-expression on piRNA levels, and show a comparison with the starting *yw* stock. The subsequent four columns show the impact of Piwi or/and Aub knockdown; note that DCR2 is present in all four of these lines. Over-expression of DCR2 does appear to impact roo piRNA levels, but has no other phenotype we have observed. The HeT-A 2801 probe, which recognizes a piRNA population that is not extensively ping-pong amplified (see Supplement Table 2), shows only a slight decease in piRNA level in the Aub knockdown lines. In contrast, the probes recognizing AT-chX-1 and Aub-bound

roo, which hybridize extensively with ping-pong amplified piRNAs (Supplement Table 2), show a significant depletion of piRNA levels on Aub knockdown. For AT-chX-1 and Aub-bound roo, similar results are seen in the Piwi-Aub double knockdown ovaries, supporting the hypothesis that the increased signal for these piRNAs in Piwi depleted ovaries (Fig. 2b) is derived from ping-pong amplified piRNAs. However, for HeT-A 2801 probe the piRNA level still shows a slight increase in the Piwi-Aub double knockdown ovaries when compare to the wt control; the reasons are not apparent at present, and further investigation will be required to resolve this issue. **b**, Comparison of the impacts of Piwi knockdown and Aub knockdown on HeT-A piRNA levels using two different northern probes. In contrast to HeT-A 2801 probe, ΔBB2, which hybridizes with piRNAs derived from ~1.5 kb of the 3'-UTR of HeT-A, recognizes a piRNA population that is strongly amplified by the ping-pong cycle (see Supplement Table 2). A significant depletion of this piRNA is observed on Aub knockdown but not Piwi knockdown, confirming the role of Aub in secondary piRNA generation from this transposon. The HeT-A 2801 probe, which hybridizes with a piRNA population that is not extensively ping-pong amplified (Supplement Table 2), did not show this behavior.

Supplement Figure 5. Germline Piwi knockdown results in loss of HP1a enrichment at the internal region of transposons. HP1a ChIP-qPCR analysis of the impact of germline Piwi knockdown on ovarian tissue using primer pairs amplifying the internal regions of the transposons. The enrichment levels are

normalized using the alpha-actinin locus. Bars represent the mean ± SEM of three biological replicate experiments.

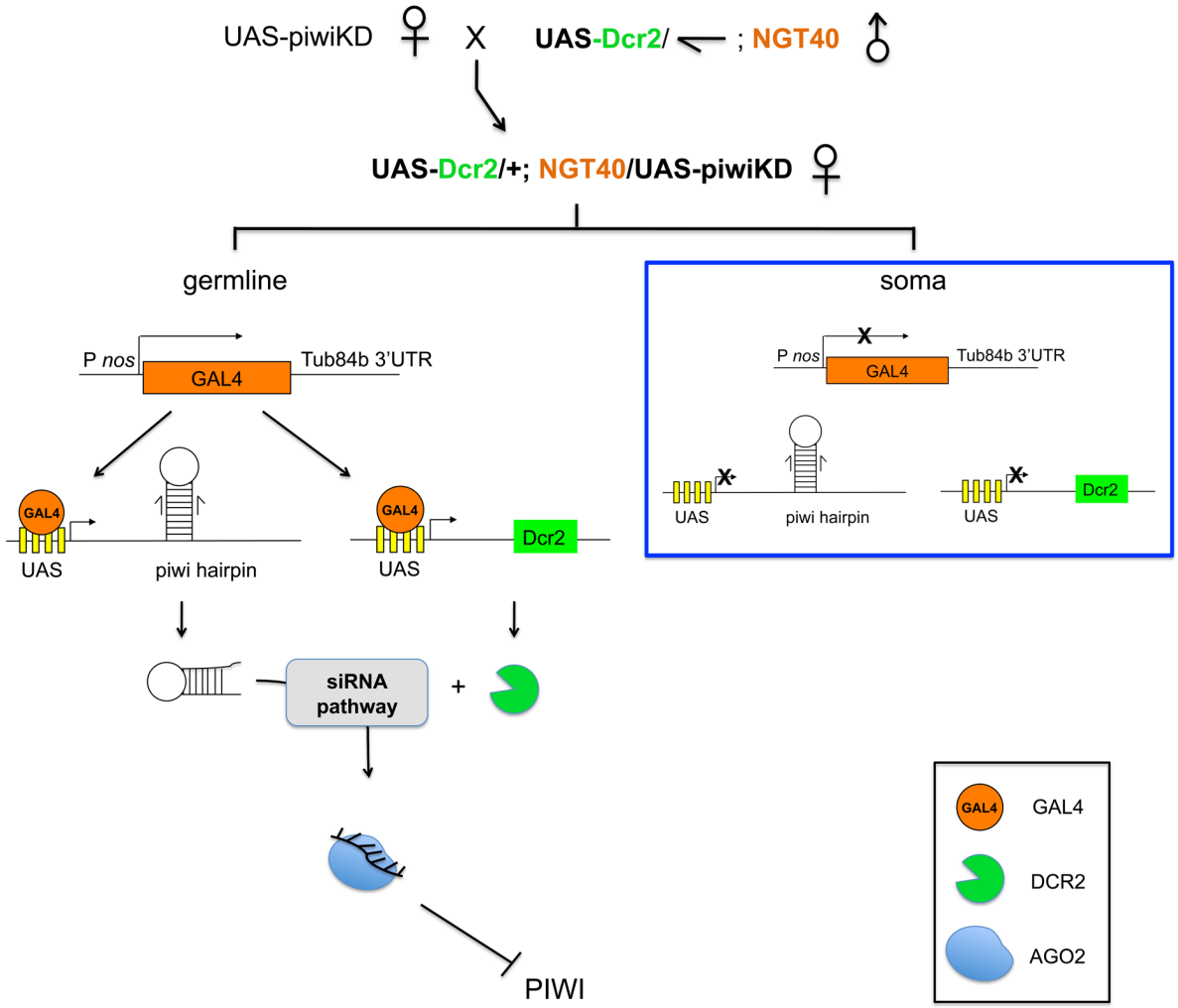
Supplement Figure 6. Germline Aub knockdown results in a significant loss of HP1a enrichment at a subset of transposons. ChIP-qPCR analysis of the 5' / promoter regions of of a set of transposons using antibodies against HP1a to assess the impact of germline Aub knockdown on ovarian tissue. The enrichment levels are normalized using the alpha-actinin locus. Bars represent the mean ± SEM of three biological replicate experiments.

Supplement Figure 7. Germline HP1a knockdown blocks oogenesis, but incomplete penetrance and varying expressivity allows assessment of the impact on transposon expression in the germline. a, Germline HP1a knockdown blocks oogenesis. Left: a representative picture of a pair of ovaries exhibiting a fully expressed HP1a knockdown phenotype. Bundles of germarium are seen, but no developed egg chambers. Right: a confocal image showing DAPI staining of the same material. **b,** HP1a immunostaining showing the efficacy of germline HP1a knockdown (control on left, mutant on right). On the right of the $HP1a^{KD}$ specimen, a string of egg chambers displays HP1a depletion with diminished HP1a staining in the germline nuclei (red arrow). An adjacent latter stage egg chamber shows residual HP1a staining (green arrow) in the germline nucleus, highlighting the incomplete penetrance and expressivity. **c,**

The fertility assay indicates that ~60 % of the females show complete expressivity of the germline HP1a knockdown phenotype.

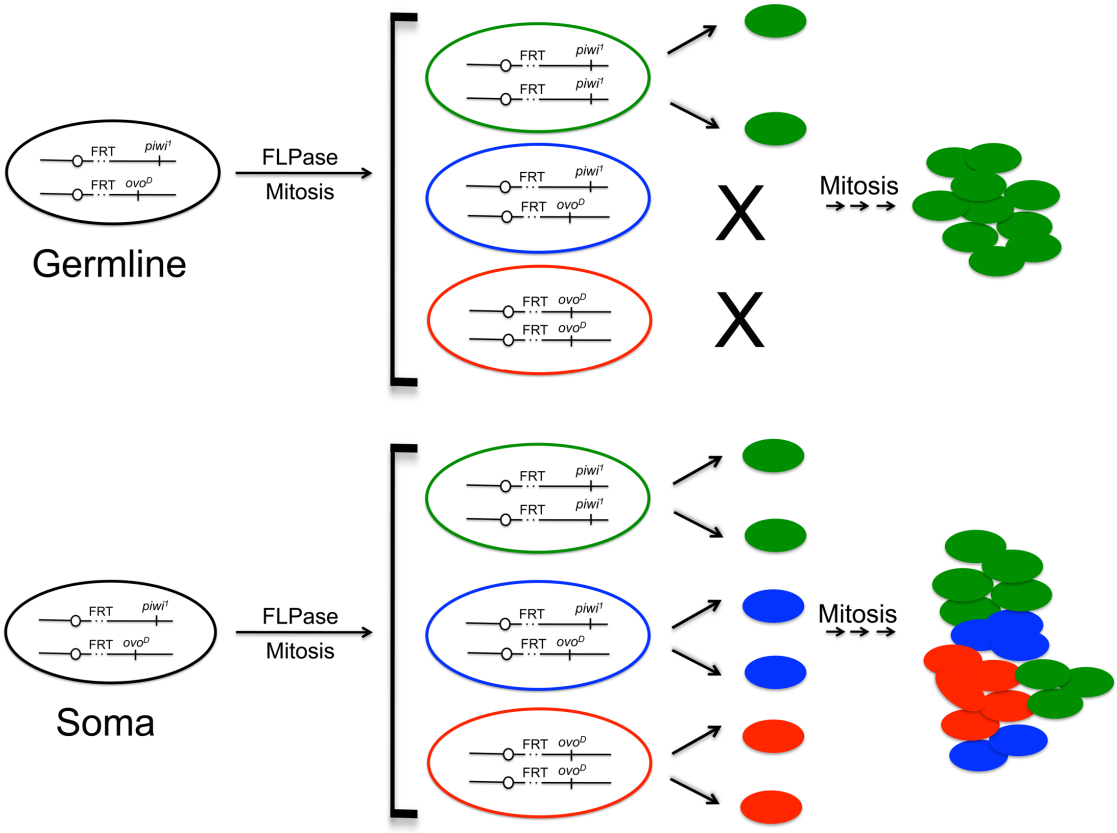
Supplement Figure 8. A model for a germline Piwi function: piRNA targeting of heterochromatin to silence transposons

Supplement Figure 1



Supplement Figure 2

a



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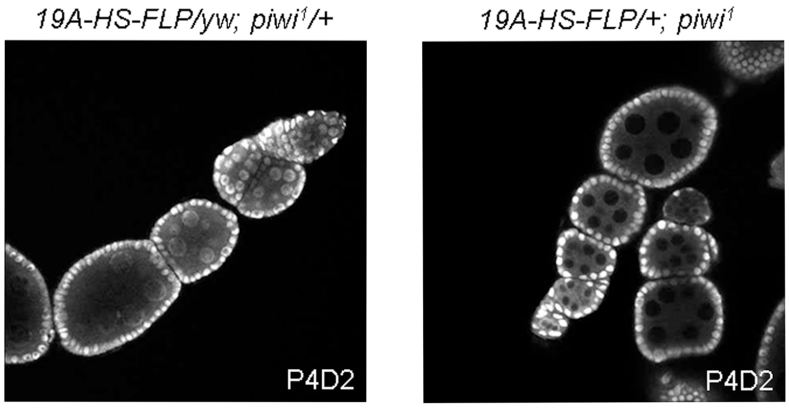
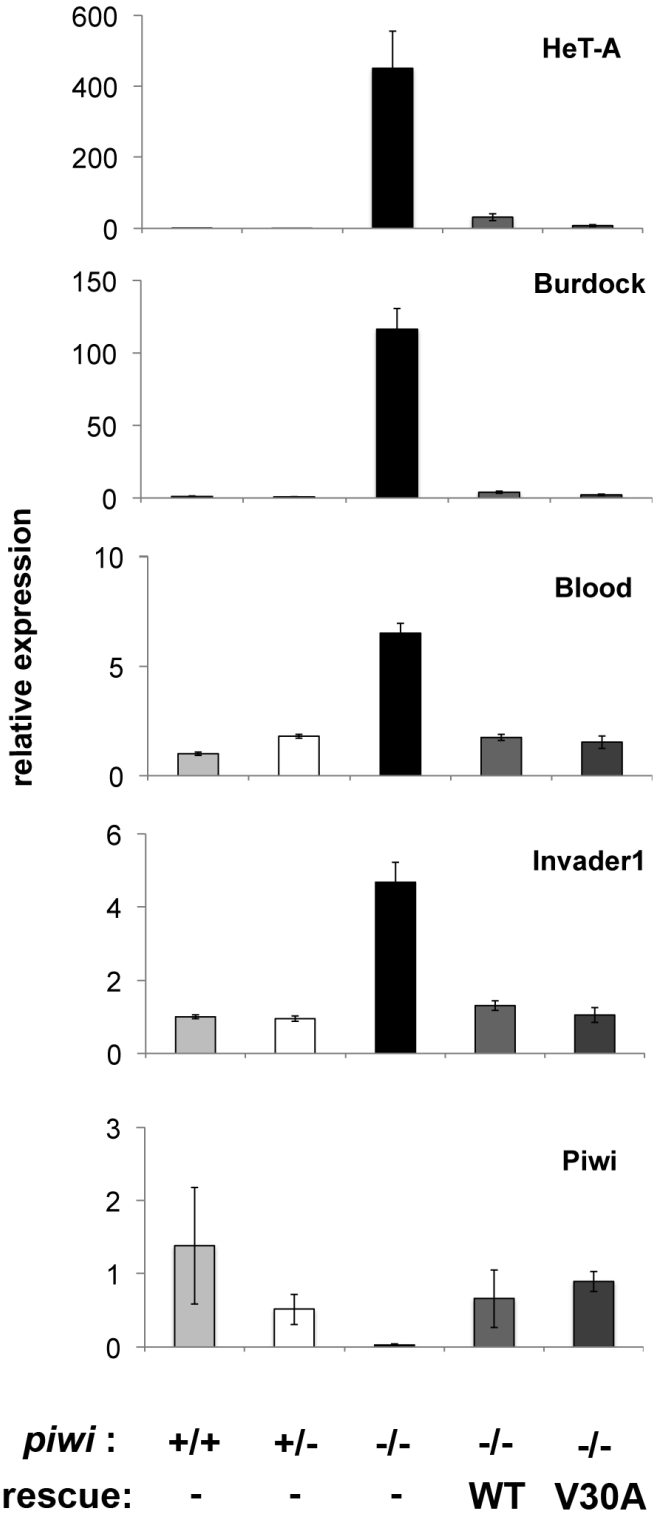


Supplement_Figure 3



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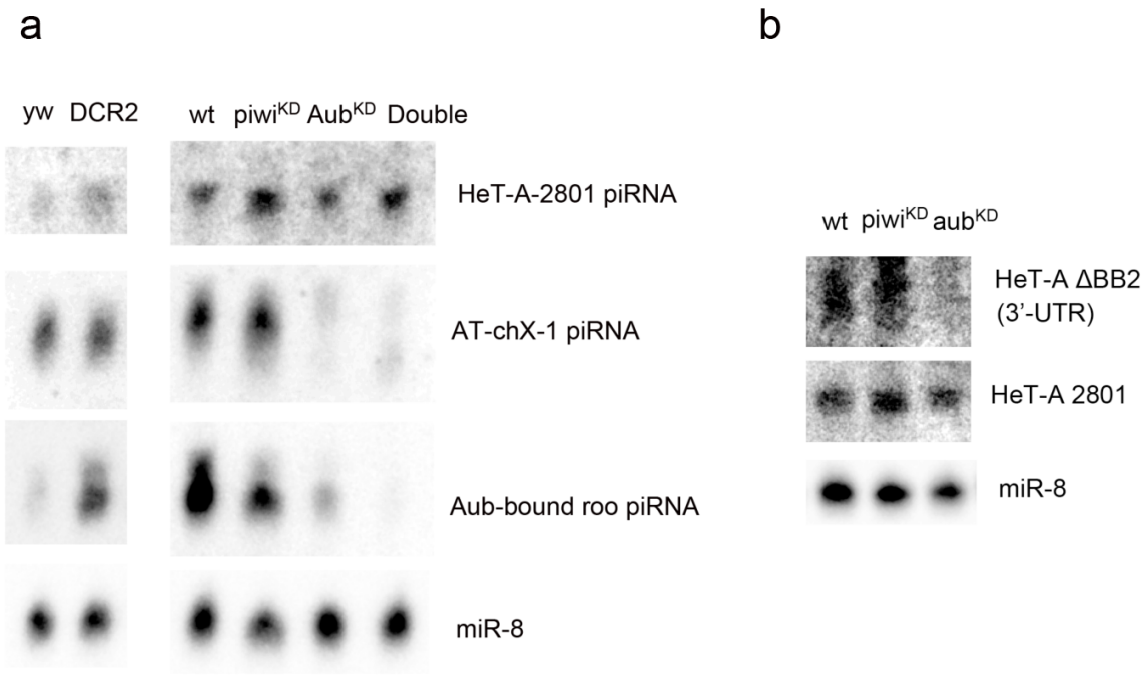
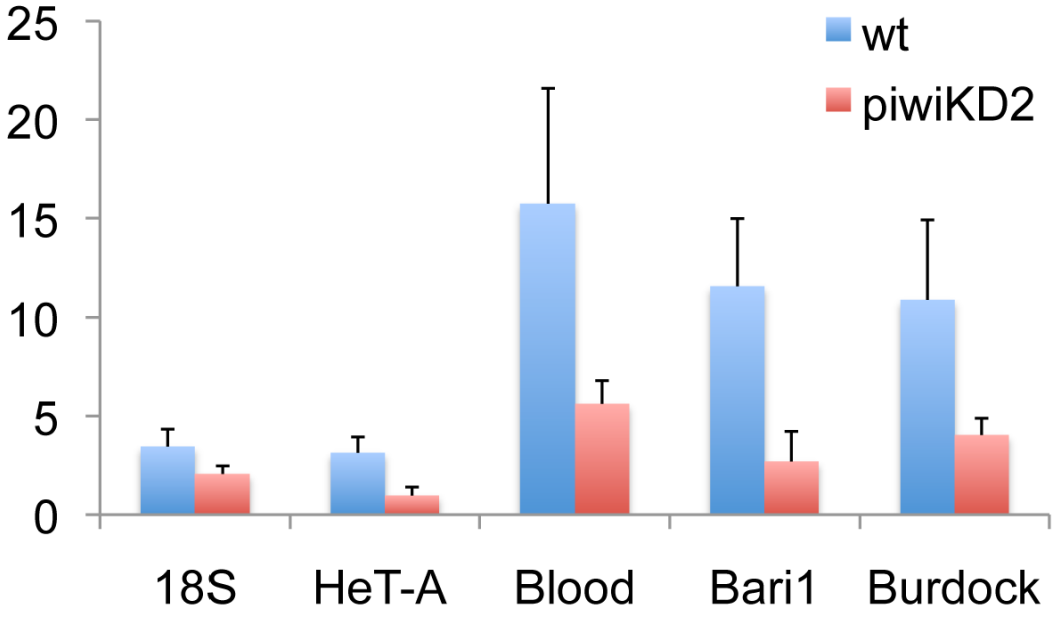


Supplement Figure 5



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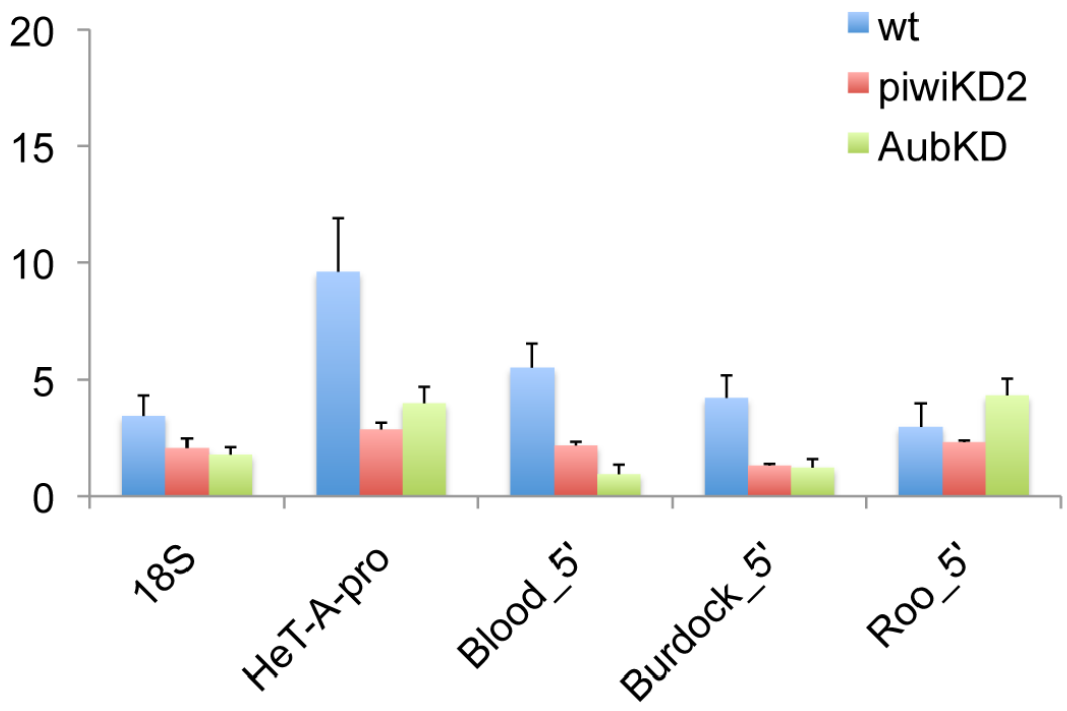

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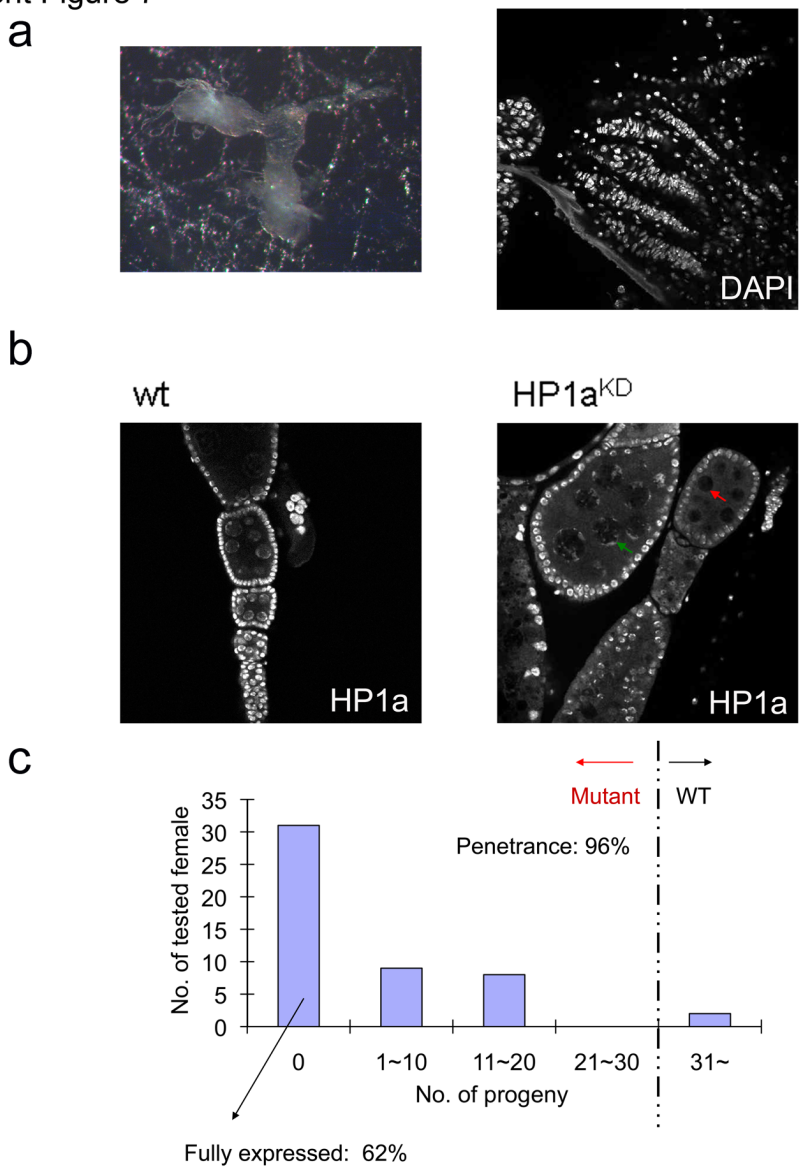
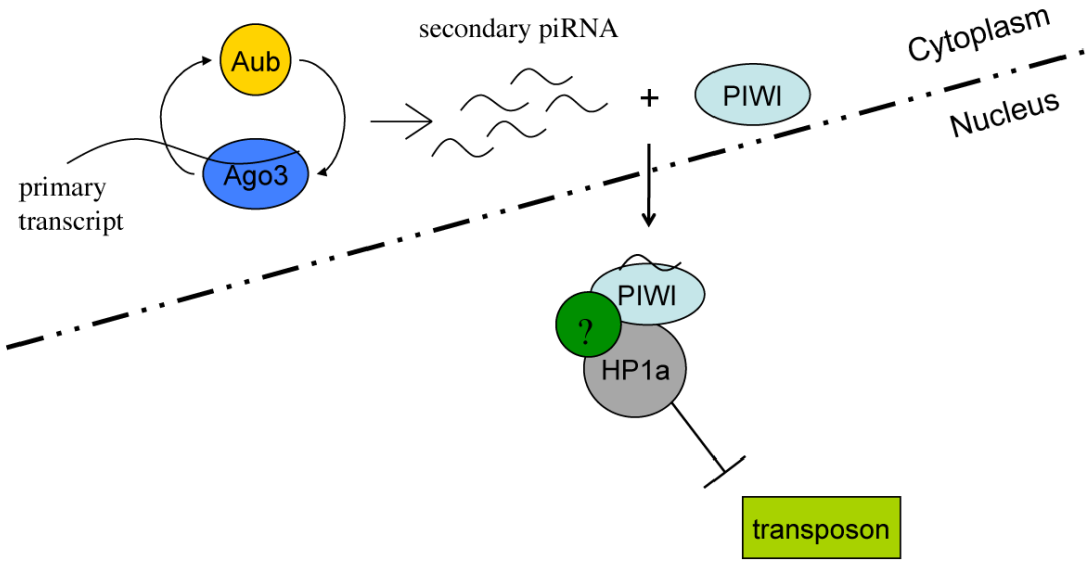


Supplement Figure 8



Supplement Table 1: Fertility test for germline Piwi knockdown females

	No. of progeny	percentage of tested females
wt N = 26	0	0
	1~5	7.69%
	6~10	11.54%
	11~20	23.08%
	20+	57.69%
piwiKD1 N = 46	0	100.00%
	1~5	0
	6~10	0
	11~20	0
	20+	0
piwiKD2 N =102	0	100.00%
	1~5	0
	6~10	0
	11~20	0
	20+	0

Supplement Table 2: Extent of ping-pong amplification identified by northern probes

Probe Name	HeT-A 2801[a]	ΔBB2 [a] (HeT-A 3'-UTR)	AT-ChX-1	Aub_roo	miR-8
Total small RNA match count [b]	0	439	2896	31	627
matches that have ping-pong partner [c]	0	241	2780	30	0
% piRNA with ping-pong partner [d]	N.A.	54.9%	96.0%	96.8%	0

a. HeT-A 2801 probe recognizes a region correspond to nt 2801-2820 of a HeT-A consensus sequence (Acc# U06920), while the ΔBB2 probe hybridizes to nt 4851-6481. The two probes have no overlapping target regions.
b. The total small RNA match count is generated by finding reverse complement reads to the probe sequence in the *ago3* heterozygote small RNA sequencing libraries generated by the Zamore lab (Li et al, 2009), one mis-match is allowed by the matching algorithm.
c. For each piRNA match, we looked into the same library to find potential ping-pong partners (using the 5' 10 nucleotide overhang criteria). piRNA reads with at least one ping-pong partner found are counted as potentially produced from ping-pong amplification
d. To estimate the extent of ping-pong amplification for the piRNA populations detected by each probe, the ratio is calculated using piRNAs with ping-pong partners (to represent the percentage of piRNAs potentially produced by the ping-pong cycle). No reads are found in the library that match the HeT-A 2801 probe and therefore a percentage cannot be estimated.

Supplement Table 3: List of fly lines used in this study

Stock NO.	Genotype	Source
BL25751	P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mC]=GAL4-nos.NGT}40	Bloomington
BL2121	P{w[+mC]=ovoD1-18}2La P{w[+mC]=ovoD1-18}2Lb P{ry[+t7.2]=neoFRT}40A/Dp(?;2)bw[D], S[1] wg[Sp-1] Ms(2)M[1] bw[D]/CyO	Bloomington
BL5133	P{ry[+t7.2]=hsFLP}1, P{w[+mC]=tubP-GAL80}LL1 w[*] P{ry[+t7.2]=neoFRT}19A; Pin[Yt]/CyO	Bloomington
V30125	w^{1118}; P{GD11831}v30125	VDRC
V31995	w^{1118}; P{GD12524}v31995	VDRC
V22235	w^{1118}; P{GD11827}v22235	VDRC
	yw; +/+; P{my^{+}=UAS-PIWIhp^{8}}	This study
	yw^{67c23}	Lab stock
	w^{1118}, $piwi^{+t6.8}$	Cox et al
178.50 A3	w^{1118}, $piwi^{V30A}$	Brower-Toland et al
	w; $piwi^{1}$, P{ry[+t7.2]=neoFRT}40A/ CyO	Cox et al

Supplement Table 4: List of in-house generated Piwi hairpin lines

Hairpin Stock	Chromosome	Homozygous	Genotype
piwihp#1	X	viable	P{my^{+}=UAS-PIWIhp^{1}}
piwihp#2	3	sterile	P{my^{+}=UAS-PIWIhp^{2}}/TM3
piwihp#3	2	lethal	P{my^{+}=UAS-PIWIhp^{3}}/CyO
piwihp#4	2	viable	P{my^{+}=UAS-PIWIhp^{4}}
piwihp#5	X	viable	P{my^{+}=UAS-PIWIhp^{5}}
piwihp#6	2	lethal	P{my^{+}=UAS-PIWIhp^{6}}/CyO
piwihp#8	3	viable	P{my^{+}=UAS-PIWIhp^{8}}

Supplement Table 5: Abbreviation key for fly genotypes

Abbreviation	Fly Genotype	Note
wt (DCR2)	UAS-DCR2/yw; NGT40/+	"Wild type" control for knockdown experiments*
piwiKD1	UAS-DCR2/w^{1118}; NGT40/ P{GD11827}v22235	
piwiKD2	UAS-DCR2/yw; NGT40/+; P{my^{+}=UAS-PIWIhp^{8}}/+	
aubKD	UAS-DCR2/w^{1118}; NGT40/ P{GD11831}v30125	
HP1aKD	UAS-DCR2/w^{1118}; NGT40/ P{GD12524}v31995	
Double	UAS-DCR2/ w^{1118}; NGT40/ P{GD11831}v30125; P{my^{+}=UAS-PIWIhp^{8}}/+	Piwi Aub double knockdown
$piwi^{1}/piwi^{1}$	19A-GAL80, hs-FLP /+; 40A-$piwi^{1}$/40A-OVO^{D}	Homozygous $piwi^{1}$ in the germline
$piwi^{1}$/+	19A-GAL80, hs-FLP/yw; 40A-$piwi^{1}$/+	

*Except for the ChIP experiment, in which UAS-DCR2; NGT40 line is used as wt control.

Supplement Table 6: Sequence of Primers used in qPCR

Name	Oligo Sequence*
297_fw	AAA GGG CGC TCA TAC AAA TG
297_rv	TGT GCA CAT AAA ATG GTT CG
jockey_fw	TGC AGT TGT TTC CCC TAA CC
jockey_rv	AGT TGG GCA AAT GCT AGT GG
INE-1_fw	GGC CAT GTC CGT CTG TCC
INE-1_rv	AGC TAG TGT GAA TGC GAA CG
Blood_fw	TGC CAC AGT ACC TGA TTT CG
Blood_rv	GAT TCG CCT TTT ACG TTT GC
S-element_fw	TGA AAA GCG TCA TTC ATT CG
S-element_rv	TGT TTC TAG CGC ACT CAA CG
Doc_fw	GGG TGA CTA TAA CGC CAA GC
Doc_rv	GCA AAA TCG ATC AGG TCT GG
1731_fw	AGC AAA CGT CTG TTG GAA GG

1731_rv	CGA CAG CAA AAC AAC ACT GC
F-element_fw	GCT GGT AGA TAC CGC TGA GG
F-element_rv	GTA GTC GTC CTC CGT TTT CG
412_fw	CAC CGG TTT GGT CGA AAG
412_rv	GGA CAT GCC TGG TAT TTT GG
NOF_fw	AGT TGG ACC TGG AAT TGT GG
NOF_rv	AAT GCA CAC GGA AGA GGA AC
Idefix_fw	AAC AAA ATC GTG GCA GGA AG
Idefix_rv	TCC ATT TTT CGC GTT TAC TG
Accord_fw	ACA ATC CAC CAA CAG CAA CA
Accord _rv	AAA AGC CAA AAT GTC GGT TG
Diver_fw	GGC ACC ACA TAG ACA CAT CG
Diver_rv	GTG GTT TGC ATA GCC AGG AT
Diver2_fw	CTT CAG CCA GCA AGG AAA AC
Diver2_rv	CTG GCA GTC GGG TGT AAT TT
Gtwin_fw	TTC GCA CAA GCG ATG ATA AG
gtwin _rv	GAT TGT TGT ACG GCG ACC TT
gypsy6_fw	GAC AAG GGC ATA ACC GAT ACT GTG GA
gypsy6_rv	AAT GAT TCT GTT CCG GAC TTC CGT CT
Hopper_fw	GGC TGG CTT CAA CAA AAG AA
Hopper _rv	GGA CTC CCG AAA ACG TCA TA
Invader1_fw	GTA CCG TTT TTG AGC CCG TA
Invader1_rv	AAC TAC GTT GCC CAT TCT GG
Max_fw	TCT AGC CAG TCG AGG CGT AT
Max _rv	TGG AAG AGT GTC GCT TTG TG
R1A1_fw	AAT TCC CGA GCT GTG CTA GA
R1A1_rv	GTC TCA AGG CAC CTT TCA GC
Rt1a_fw	CCA CAC AGA CTG AGG CAG AA
Rt1a_rv	ACG CAT AAC TTT CCG GTT TG
springer upper	CCA TAA CAC CAG GGG CA
sprinter lower	CGA GTG CTG GTC TGT CA
aurora upper	GCC ATC ACC GAC TAC CAT TA
aurora lower	TCA CCC CAT CCG ACG CTC TT
opus upper	CGA GGA GTG GGG AGA GAT TG
opus lower	TGC GAA AAT CTG CCT GAA CC
Bari-1 upper	CAC ACC AAC CAA CAA AAC A
Bari-1 lower	AAA GAA ATA CGA AAG ATA AC
Bari1_fw_1	TTG AAA ACG TTT GGG CTT TT
Bari1_rv_1	TAA CAC CAC CTT TGG CAT CA
Bari1_fw_2	CAT GGG TCA CAA ACA GTT GC
Bari1_rv_2	TTC ATT TGC CTC TTC CTT GC
1360_fw_1	GCG TAA CGC CAT ACG ATT TT
1360_rv_1	CGG TGA AGG GCA TAT TTT GT
1360_fw_2	TGT TGA TGG CTT GAT CCA AA
1360_rv_2	CGC AAT TCG ACT TTT GTG AA

Invader 4_fw_1	TAA TGC GCT TGT AGG CAG TG
Invader 4_rv_1	AGC TCT CTC CAG GTG TTG GA
Invader 4_fw_2	TGA GAG AAG ACA CGC CAA TG
Invader 4_rv_2	TGT GCC TAA TCC ACC AAT CA
Burdock_fw	CGG TAA AAT CGC TTC ATG GT
Burdock_rv	ACG TTG CAT TTC CCT GTT TC
cop-s	GCA TGA GAG GTT TGG CCA TAT AAG C
cop-as	GGC CCA CAG ACA TCT GAG TGT ACT ACA
Het-s2	CGC AAA GAC ATC TGG AGG ACT ACC
Het-as2	TGC CGA CCT GCT TGG TAT TG
roo s	CGT CTG CAA TGT ACT GGC TCT
roo as	CGG CAC TCC ACT AAC TTC TCC
mdg1 s	AAC AGA AAC GCC AGC AAC AGC
mdg1 as	CGT TCC CAT GTC CGT TGT GAT
RpL32_f	CGA TCT CGC CGC AGT AAA C
RPL32_ rv	CTT CAT CCG CCA CCA GTC G
PIWI-RT-for	ACTTCCCGAGGTAGTGGTGA
PIWI-RT-Rev	CGGTTCCCTTCTTGGATACC
Aub rt primer2 f	GAC GTG GTC GAG GAA GAA AG
Aub rt primer2 Rv1	GCG CTC AAA CTG GAT TTC TC
Aub rt primer2 Rv2	TAT CGA GGG ACC CAC TTG AG
HetA promoter FW	ACCACGCCCAACCCCCAA
HetA promoter RV	GCTGGTGGAGGTACGGAGACAG
alpha-actinin-1	CAGCAAGCACCTCTGCTCTA
alpha-actinin-2	TGCAAGCGTATGTGAGATCC
Blood_5'_fw	TTATTACATGGCGACCGTGA
Blood_5'_rv	CTCACACCTGTTGTCGCTGT
Invader1_5'_fw	GAAGAGGAAGAAGGGCATCC
Invader1_5'_rv	AATGGCGATTTTGCGACTCC
Roo_5'_fw	CCTCTGCGTAGGCCATTTAC
Roo_5'_rv	AAGGCTCGATTGACCAAATG
Burdock_5'_fw	ATTAGAAGCGTCGGTCATCG
Burdock_5'_rv	GGGCGCCAATTATCATTTTA
Jockey_5'_fw	ATGGGAGATGAGCAATCGAG
Jockey_5'_rv	GGGCAAAAACAACACAGCTT
18S_rDNA_1	TTCATGCTTGGGATTGTGAA
18S_rDNA_2	GTACAAAGGGCAGGGACGTA

*Li et al, 2009; Klenov et al, 2007; Specchia et al, 2010.

Supplement Table 7: Sequence of probes used in piRNA Northern

Name	Sequence*
HetA-2801	TGC GGC ACC CTG TGT CCC GG
Aub-bound roo piRNA	AAGAAATCAGTAATGCACTCTAGTA
AT-chX-1 piRNA	GCCCGAGCCGTCTAACGATGAAACA
miR-8	GACATCTTTACCTGACAGTATTA
HeT-A ΔBB2	Position 4851-6481 of λ23Zn (Acc# U06920)

*Li et al, 2009; Nishida et al, 2009; Danilevskaya, 1994.

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Chapter 5

Genetic interactions among piRNA pathway components reveal multiple modes of transposon silencing in the *Drosophila melanogaster* female germline

Genetic interactions among piRNA pathway components reveal multiple modes of transposon silencing in the *Drosophila melanogaster* female germline

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Abstract

Piwi-interacting RNA (piRNA) plays a major role in silencing transposable elements (TEs) in animal reproductive systems. Previously we reported a role for Drosophila Piwi in transposon silencing in the female germline downstream of secondary piRNA production, involving recruitment of HP1a to a subset of TEs. We also found that certain TEs appear to require Piwi, but not secondary piRNA production (identified as a requirement for *aubergine*), for regulation. Here, we extend our analysis to the additional piRNA pathway components Armitage and Squash. We find that while Armitage works with Piwi in regulating most transposons, Squash appears to function specifically in secondary piRNA-dependent silencing. Analysis using a Piwi N-terminal truncation mutation suggests that both secondary piRNA dependent and independent silencing requires nuclear localization of Piwi; the only exception for this requirement is the TE *Jockey. piwi, aub* double knockdown reveals complex genetic interactions, indicating different dependencies for different TEs. The redundancy observed between *piwi* and *aub* in *Jockey* silencing further supports a cytoplasmic role for Piwi in this case. We conclude that Piwi can function independently of secondary piRNA production by Aub and propose that it does so either by utilizing primary piRNA directly or by taking the place of Aub in the ping-pong cycle.

Introduction

Transposons are selfish DNA elements that propagate by invading the reproductive system (germline DNA) of their host (Werren, 2011). Mobilization of a transposable element can lead to mutagenic events, as a new insertion of the element can lead to disruption of a coding exon or a regulatory element. Although in some instances, a symbiotic relationship can develop between the host and the mobile element (Beauregard et al., 2008), transposition events in general have negative impacts on the fitness of the host. Since transposons and repeats can take up a large portion of the host genome (Lander et al., 2001), mechanisms to keep these elements in check must be in place to ensure the survival of the host species.

In animals, the PIWI family argonaute proteins, Piwi, Aub and AGO3, work together to provide a major mechanism for transposon control (Saito and Siomi, 2010). PIWI proteins are most abundantly expressed in the reproductive system and are associated with a novel class of small RNAs, piRNAs (PIWI-interacting RNAs) (Saito et al., 2006; Brennecke et al., 2007; Yin and Lin, 2007). piRNAs are enriched in sequences derived from TEs, and show characteristic features that set them apart from the other classes of small RNA. One important feature is its suggested mode of biogenesis, which does not involve an RNaseIII Dicer protein (Vagin et al., 2006). The production of piRNA is best characterized in the fruit fly, *Drosophila melanogaster* (Senti and Brennecke, 2010). Studies there indicate that piRNA production in the female germline begins with long transcripts produced by RNA polymerase II from discrete loci (piRNA clusters). These

transcripts then get processed into small RNAs (primary piRNAs) through a mechanism (not yet fully understood) involving a putative nuclease, Zucchini (Saito et al., 2009, 2010; Haase et al., 2010). Primary piRNAs are fed into an amplification loop (Ping-Pong cycle) involving the PIWI family proteins to generate secondary piRNAs from transcripts of TEs (of matching sequence) (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009). The 5' end of the secondary piRNAs is produced by either Aub or AGO3 slicing, but the mechanism for 3' end generation remains unclear (Kawaoka et al., 2011). The process of piRNA production in the ovarian soma is thought to be similar to the germline process, except that there is no secondary amplification step (Malone et al., 2009; Saito et al., 2009).

The germline ping-pong amplification cycle described above for secondary piRNA biogenesis has direct implications for how piRNA acts in suppressing retrotransposons. RNA intermediates, which are required for retrotransposon mobilization, have been proposed to be the source of the sense strand piRNA (Brennecke et al., 2007; Gunawardane et al., 2007). Slicing of the RNA intermediate by anti-sense piRNA-guided Aub generates the 5' end of a sense piRNA and simultaneously eliminates the transposition potential of the RNA intermediate (Brennecke et al., 2007; Gunawardane et al., 2007). This scenario bears some similarity to the post-transcriptional silencing mechanism used in the siRNA pathway (Carthew and Sontheimer, 2009). Aub thus can function in secondary piRNA production while at the same time suppressing

retrotransposons through its slicing activity. This silencing mechanism, executed by slicing RNA transcripts from various sources, operates in the cytoplasm.

The role for Piwi in transposon silencing appears to be more complex. The nuclear localization of Piwi makes it distinct from the other two PIWI family members, Aub and AGO3 (Cox et al., 1998; Brennecke et al., 2007; Gunawardane et al., 2007). The lack of an Aub/ AGO3 ping-pong amplification cycle in the ovarian soma simplifies the study of piRNA biogenesis and function there (Malone et al., 2009; Saito et al., 2009). Using ovarian somatic cell lines, the functions of the individual domains of Piwi have been analyzed (Saito et al., 2009, 2010). Both the capacity to bind piRNA (PAZ domain) and an intact N-terminal domain are essential for nuclear localization and transposon silencing by Piwi (Saito et al., 2009, 2010). A PAZ domain mutation disrupts Piwi-piRNA interactions and leads to a cytoplasmic localization pattern for Piwi (Saito et al., 2010). Similarly, RNAi knockdown of Armitage, a putative helicase, in this cell line also leads to a disruption of piRNA loading and results in a cytoplasmic localization pattern for Piwi (Saito et al., 2010). It remains to be determined whether Armitage performs similar functions in the germline. Studies using the mutant allele $piwi^{Nt}$ (which produces a protein lacking the N-terminal 26 amino acids) in flies also show an association between loss of Piwi nuclear localization and loss of transposon silencing (Klenov et al., 2011). Nonetheless, it appears that low amount of Piwi proteins normally localize in the cytoplasm of germline

cells (Brennecke et al., 2007), and it remains to be determined whether cytoplasmic Piwi can play a role in silencing some (not yet tested) transposons.

How Piwi executes transposon silencing in the nucleus is an active area of research. Previously, reports documenting an interaction between Piwi and HP1a have suggested a chromatin-based transcriptional silencing mechanism (Brower-Toland et al., 2007; Mendez et al., 2011). Recently three groups have independently reported functional analyses supporting such a model (Klenov et al., 2011; Shpiz et al., 2011; Wang and Elgin, 2011). It has been shown that lack of germline Piwi, or simply a lack of nuclear Piwi in the ovaries, impacts the chromatin structure at transposon sites, resulting in a loss of HP1a where it is normally enriched (Klenov et al., 2011; Wang and Elgin, 2011). Germline HP1a depletion has impacts on transposon expression, showing an increase comparable to that seen on germline Piwi knockdown (Wang and Elgin, 2011). A run-on analysis performed in a study of the telomeric retrotransposons confirmed that the impact is on the level of transcription (Shpiz et al., 2011). Thus, it appears that in addition to a post-transcriptional silencing mechanism via Aub, piRNA can also mediate a transcriptional silencing mechanism through Piwi-dependent HP1a recruitment.

We have previously shown that, in the female germline, HP1a recruitment to TEs via Piwi is a process downstream of Aub/ AGO3 secondary piRNA production (Wang and Elgin, 2011). However, we also noticed that some

transposons require Piwi, but neither Aub nor AGO3, for proper silencing in the germline. These observations point to an additional Piwi-dependent silencing pathway, one that functions in the germline but does not require secondary piRNA. We now report that depletion of Armitage or Squash in the germline recapitulates the dichotomy in the transposon expression profile observed on depletion of Piwi or Aub. Experiments utilizing the mutant allele *piwi*^{Nt} indicate that both pathways (secondary piRNA-dependent and –independent) depend on Piwi function in the nucleus. In contrast, the transposon Jockey appears to be silenced by a mechanism that utilizes Piwi in the cytoplasm. Finally, double knockdown of *piwi* and *aub* reveals genetic interactions between the secondary piRNA dependent and independent pathways in the germline.

Results

Germ line depletion of Aub impacts Piwi nuclear localization and suggests a role for Piwi in transposon silencing that occurs downstream of secondary piRNA production (Wang and Elgin, 2011). However, some transposons require Piwi but neither Aub nor AGO3 for silencing, suggesting that Piwi can also function independently of secondary piRNA (Wang and Elgin, 2011). The LTR retrotransposons *Invader1* and *Max* showed such properties (Table 1). Interestingly a LINE-like element, *Jockey*, requires Piwi and AGO3 for silencing, but not Aub (Table 1). The lack of involvement of AGO3 and Aub in Invader1 and Max silencing is reminiscent of the somatic piRNA pathway (Malone et al., 2009;

Saito et al., 2009). In the ovarian soma, Piwi is the sole PIWI family argonaute protein expressed and operates a piRNA pathway without AGO3 and Aub contributions. We speculate that a similar mechanism could operate in the germline to silence Invader1, Max and similar transposons. To look for further evidence supporting a germline pathway independent of secondary piRNA production, we screened additional genes thought to be involved this pathway. Two independent studies have reported on proteins that co-immunoprecipitate with Piwi in extracts from cell lines derived from ovarian soma (Haase et al., 2010; Saito et al., 2010). Drawing on these results, we selected Armitage (a putative helicase) and Squash (a putative nuclease) as the most likely partners in this secondary piRNA independent pathway to further test their roles in germline transposon silencing, particularly examining Invader1 and Max.

To test their functions in the germline, we used a *Nanos* driver NGT40 to drive germline-specific RNAi knockdown to silence gene expression at the transcript level. In conjunction with the hairpin construct, a DCR2 over-expression construct is used in order to achieve potent depletion of the target mRNA (Wang and Elgin, 2011). Quantitative assessment of Squash and Armitage expression levels using whole ovaries indicates that while Squash knockdown depletes about 60% of the *squash* product, Armitage knockdown leads to more than 70% depletion for both isoforms of *armitage* (Figure 1a). Since approximately one third of the ovary is composed of somatic follicle cells,

we infer that both the knockdowns performed here deplete most transcripts in the germline.

We then determined expression levels for transposons in ovaries depleted of germline Armitage or Squash, respectively. We focussed on a set of TEs that were shown previously to respond strongly to germline depletion of both Piwi and Aub, and a set of those that respond only to depletion of Piwi (Figure 1b). Similarly to what was observed for both Piwi and Aub, we find strong over-expression of Blood, Burdock and HeT-A in both Squash and Armitage germline knockdown ovaries. Over-expression is seen to a lesser extent for transposons 1731 and Invader4. And several transposons that showed differential responses to Piwi or Aub knockdown also show differential responses to Armitage and Squash knockdown. In the case of Invader1, no impact on expression is observed following either Armitage or Squash germline knockdown. In the cases of transposons Max and Jockey, germline knockdown of Squash shows no significant impact on their expression, but ~4 fold over-expression is observed for both transposons in germline Armitage knockdown ovaries (Figure 1b). Interestingly, when comparing transposon expression profiles among lines with germline knockdowns of Piwi, Aub, Squash or Armitage, we found that while Squash knockdown lines show a response profile similar to Aub knockdown lines for every transposon tested, Armitage knockdown lines show a response profile similar to germline Piwi knockdown lines with one exception, Invader1 (Table 2). We interpret the similarity in transposon expression profiles between the different

knockdown lines as indicative of functional relationships between these RNAi-component genes in their normal context. We therefore infer that Squash is likely involved in the secondary piRNA-dependent silencing pathway, while Armitage functions with Piwi in both secondary piRNA-dependent and -independent pathways.

Squash is a novel RNase HII-like protein reported to function in piRNA-mediated transposon silencing (Pane et al., 2007). However, mutations in Squash have no significant impact on piRNA production or Piwi localization (Malone et al., 2009; Haase et al., 2010). The similarities observed here between the consequences of Aub and Squash germline knockdown indicate a functional link between the two proteins. Previously, Pane et al. (2007) also reported potential interactions between Squash and Aub using transgenic constructs in a co-immuno-precipitation experiment. Taken together with the observation of co-immunoprecipitation of Piwi and Squash (Haase et al., 2010), the evidence suggests that Squash could function in mediating a transition of secondary piRNAs between Piwi and Aub. We therefore looked for an overlap between secondary piRNA populations that interact with Piwi and those that interact with Aub. Piwi- and Aub-interacting small RNAs have previously been shown to share common features such as enrichment for antisense transposon sequences and similar size distributions (Brennecke et al., 2007). To look for evidence of a transition of piRNAs between the two PIWI family proteins, we considered the percentage of perfectly matching piRNAs between Piwi- and Aub- interacting

small RNAs, analyzing the small RNA sequencing datasets previously published by Brennecke et al (Brennecke et al., 2007). In this analysis, secondary piRNAs are defined by the presence of a ping-pong partner. We found that about 44% of the secondary piRNAs bound by Piwi have at least one exact match in the pool of Aub-interacting piRNAs. This percentage of overlap is significant, especially given the fact that the sequencing libraries analyzed here are far from saturation (Brennecke et al., 2007). This observation further supports a model in which Piwi functions downstream of secondary piRNA production to silence this set of transposons.

Armitage is a putative helicase reported to function in loading piRNA onto Piwi in the ovarian soma (Saito et al., 2010). Consistent with such a role in the germline, Armitage knockdown shows a similar transposon expression profile when compared to germline Piwi knockdown (Table 2), except for the response of *Invader1*. While it remains unclear how *Invader1* silencing is maintained, a role for Armitage in loading piRNA onto Piwi in the germline can explain its involvement in both secondary piRNA-dependent and -independent transposon silencing. piRNA loading is required for nuclear localization of Piwi in the ovarian soma (Saito et al., 2010). Similarly, we find that in germ cells depleted of Armitage, Piwi localization is mostly cytoplasmic; in contrast to the nuclear localization pattern observed in wildtype ovaries (Figure 1c). We therefore conclude that Armitage likely functions in piRNA loading to Piwi in the germline as well as the soma.

We have previously shown that germline Piwi can function downstream of secondary piRNA production to silence transposons in the nucleus (Wang and Elgin, 2011), however, it remains unclear whether Piwi could function in the cytoplasm for silencing certain transposons, such as *Invader1* and *Max*. In addition to piRNA loading, nuclear localization of Piwi also requires an intact N-terminal domain (Saito et al., 2009). *piwi*^{Nt} is a mutant allele which produces a stable cytoplasmic Piwi protein lacking the usual N-terminal 26 residues, replaced with MQ (Klenov et al., 2011). This protein suffices to maintain the germline stem cells in their niche, but fails to silence a set of tested transposons (Klenov et al., 2011). Here we extend the analysis of transposon expression to the set of elements studied in Figure 1. Using ovaries from homozygous *piwi*^{Nt} flies, we find that in addition to a strong impact on HeT-A and Burdock expression, Invader 1 and Max also show a ~5-fold increase in expression relative to the wildtype control (Figure 2). Interestingly, among the tested transposons, a LINE like element, Jockey, appears to be the only transposon that is silenced by the protein product of *piwi*^{Nt}. [Jockey shows ~5-fold over-expression following germline Piwi knockdown (Wang and Elgin, 2011).] This observation indicates that *piwi*^{Nt} is functional in silencing some transposons, presumably via a piRNA-mediated process. We therefore infer that Piwi can silence Jockey using a cytoplasmic mechanism in wild type flies. Given the observation that *piwi*^{Nt} is functional in Jockey silencing, the increased expression of *Invader1* and *Max* argue that silencing of these transposons is not achieved by

cytoplasmic Piwi. We further infer that the secondary piRNA-independent mechanism for transposon silencing likely also requires nuclear localization of Piwi. However, it is important to keep in mind that we are unable to distinguish between germline and somatic effects in experiments using the *piwi*^{Nt} allele, as the mutation is present in all cells.

Thus, transposon silencing by piRNA appears to utilize multiple mechanisms. In the female germline, lack of Piwi and Aub impacts some common transposon targets, while some transposons are dependent on Piwi but not Aub for suppression (Table 1). To further dissect this process, we analyzed epistatic interactions between Piwi and Aub. Using the same germline-specific knockdown strategy, we simultaneously depleted both Piwi and Aub (Figure 3a) and compared the impacts on transposon expression to the impacts of the individual knockdowns (Figure 3b). Amongst the transposons tested, we categorize the responses into four different types. First, we have transposons *Blood* and *HeT-A*, which show similar responses to either Piwi-, Aub- or double-knockdown (Figure 3b). A lack of an additive effect in a double knockdown as seen here suggests that Piwi and Aub function in the same pathway to silence these transposons. Second, we have transposon *Invader1*, which only responds to germline Piwi depletion but not Aub depletion. The double knockdown shows the same impact on *Invader1* expression as Piwi knockdown (Figure 3b). This again supports the interpretation that Piwi can function in silencing some transposns independent of secondary piRNAs. Third, for transposons *1731* and

Burdock, each Piwi and Aub knockdown shows similar impact on their expression level while the double knockdown results in a level of over expression close to the sum of impacts from each individual knockdown (Figure 3b). The additive effect is indicative of Piwi and Aub functioning in two independent pathways to silence these transposons. Finally, in the case of transposons *Invader4* and *Jockey*, one sees significant over-expression in germline Piwi knockdown and mild to no response to germline Aub knockdown. Interestingly, a strong synergistic effect is observed in the Piwi-Aub double knockdown, particularly in the case of Jockey (Figure 3b). A synergistic effect in the double knockdown suggests redundancy between the actions of Piwi and Aub. Since Aub functions in the cytoplasm, this interpretation fits well with our observation of Jockey suppression by Piwi in the cytoplasm. The double knockdown results support a model in which some transposons are silenced by a mechanism requiring Piwi and Aub to work together (for example, a pathway dependent on secondary piRNA) and other transposons are silenced by a mechanism requiring Piwi but not Aub. The suggestions of synergism hint at more complex interactions between Piwi and Aub in silencing different transposons. It is unclear how the mode of silencing for each transposon is determined. Amongst transposons showing similar responses to germline depletions of piRNA pathway components we observe no straightforward correlation in terms of known structural features.

Discussion

Observation of examples of Aub- and AGO3-independent transposon silencing in the female germline led us to the search for a mechanism for germline Piwi-dependent silencing that is independent of secondary piRNA. In the ovarian soma, Piwi is known to function in transposon silencing in the absence of Aub and AGO3 (Malone et al., 2009; Saito et al., 2009). Results from studies done in that system are therefore likely to be informative concerning a potentially parallel mechanism in the germline. However, the results presented here by studying germline Armitage and Squash, which have been shown to co-immunoprecipitate with Piwi in the ovarian soma (Haase et al., 2010; Saito et al., 2010), did not lead to an identification of components specific to the Aub- and AGO3-independent transposon-silencing pathway in the germline. Instead, we found that Armitage likely functions in both Aub dependent and independent silencing, while Squash likely functions specifically in the Aub-dependent pathway (Fig 1b and Table 2). Although a factor involved specifically in the secondary piRNA-independent pathway remains to be indentified, our results with Armitage and Squash recapitulate the dichotomy observed between Piwi and Aub in transposon silencing and are consistent with the interpretation of a secondary piRNA-independent silencing pathway in the germline.

Armitage knockdown in an ovarian somatic cell line leads to mis-localization of Piwi from the nucleus and a lack of piRNA loading (Saito et al., 2010). Similarly, in germline Armitage knockdown ovaries, we observe an

abnormal Piwi localization pattern, with Piwi enriched in the cytoplasm (Figure 1c). In addition, results from Malone et al. sequencing the small RNAs pulled down with Piwi from Armitage mutant ovaries also indicate a lack of piRNA loading (Malone et al., 2009). Taking these results together, we conclude that germline Armitage likely functions in loading piRNAs onto Piwi in both the secondary piRNA-dependent and -independent pathways. It is interesting to note that the Piwi staining pattern observed in germline Armitage knockdown ovaries presented here is distinct from the pattern reported in *armitage* mutant ovaries (Malone et al., 2009). The differences in the approach in depleting Armitage are the likely cause of this discrepancy. Describing Armitage function as loading piRNAs onto Piwi in both pathways does not provide a model that explains the lack of impact on *Invader1* expression observed in germline Armitage knockdown ovaries (Fig 1b). It is possible that the knockdown is sufficient to cause some but not all phenotypes associated with germline Armitage depletion.

Results from germline Squash or Aub knockdowns show high similarity in terms of the types of transposons affected and the degree of increase in expression of each (Table 2). It remains unclear exactly what the role of Squash is in the process. Given its putative RNaseH domain, Squash was first predicted to function as a nuclease in processing the 3' ends of piRNAs (Pane et al., 2007). However, mutations in Squash do not appear to impact the overall level or length distribution of piRNA (Malone et al., 2009; Haase et al., 2010). Our results, taken together with previously published observations on co-immunoprecipitation with

Piwi and Aub respectively, lead to a hypothetical role of Squash in the transition of piRNAs between the two PIWI proteins. This is supported by an analysis of published piRNA datasets. However, It is unclear how the putative RNaseH domain of Squash would function in this scheme. Alternatively, Squash could function in a downstream process to mediate the interaction between piRNA and its putative DNA/ RNA target in a transcriptional or co-transcriptional silencing mechanism. RNaseH functions to cleave the RNA strand in a DNA/RNA heteroduplex; by extrapolation, Squash could function in removing the piRNA, which is paired with target DNA, to identify loci for heterochromatin formation through HP1a recruitment. Despite being rather speculative, this interpretation is fully compatible with our observation that Squash is involved specifically in the secondary piRNA dependent pathway and consistent with a proposed role of Squash in the “effector phase” of the piRNA silencing pathway (Haase et al., 2010).

We previously reported the failure of Piwi nuclear localization in germline Aub knockdown ovaries (Wang and Elgin, 2011). This observation led us to speculate that in the secondary piRNA independent pathway, Piwi could function in the cytoplasm to achieve transposon silencing. Here, we test this hypothesis by examining the impacts on expression levels of *Max* and *Invader1* in mutant ovaries expressing only cytoplasmically localized Piwi. Although we are unable to distinguish between germline and somatic contributions to the detected over-expression signals, we infer that Piwi likely functions in the nucleus in both the

germline secondary piRNA independent pathway and the somatic pathway. We therefore propose that in the germline the majority of both secondary piRNA dependent and independent functions of Piwi are executed in the nucleus. Our previous observation of Aub knockdown impact on Piwi nuclear localization likely reflects only the secondary piRNA dependent Piwi, while the residual Piwi signal in the nucleus is likely coming from the secondary piRNA independent Piwi (Wang and Elgin, 2011).

LINE-like element Jockey appears to be silenced by Piwi in the cytoplasm, as the N-terminal truncated Piwi is sufficient for silencing. This interpretation is consistent with our previous observation that Piwi silencing of Jockey is independent of HP1a recruitment (Wang and Elgin, 2011). In addition, a cytoplasmic function for Piwi in Jockey silencing also fits well with the results observed in the Piwi, Aub double knockdown ovaries. The redundancy observed is better explained with the two proteins functioning in the same cellular compartment. The redundancy between Piwi and Aub also implies a slicer domain-mediated mechanism for Piwi in silencing this transposon, as was originally proposed (Brennecke et al., 2007; Gunawardane et al., 2007).

Results from the Piwi, Aub double knockdown lines appear to reveal rather complicated interactions between the two PIWI family proteins. However, by introducing a secondary piRNA independent pathway in the germline, which also utilizes Piwi, the observed additive/synergistic effects can be described by

interactions between Aub and the secondary piRNA independent Piwi at different levels (Figure 4). We propose that the secondary piRNA independent pathway described here is likely identical to the primary piRNA pathway first proposed to explain the initiation of the ping-pong amplification cycle (Brennecke et al., 2007; Gunawardane et al., 2007). We therefore infer that in addition to initiating secondary piRNA production through the ping-pong cycle, primary piRNAs can function directly in transposon silencing via Piwi in the nucleus.

Materials and Methods

Fly Lines and Husbandry

Flies were reared on regular cornmeal sucrose-based medium (Shaffer et al., 1994) and maintained at 25 °C, 70% humidity for all genetic analyses. Hairpin lines used are *yw; P{my*$^{+}$*=UAS-PIWIhp8}* (piwiKD) (Wang and Elgin, 2011) , and *w*^{1118}*;P{GD7319}v16205* (armiKD), and *w*^{1118}*; P{GD11831}v30125* (aubKD), and *w*^{1118}*;P{GD16229}v47103/TM3* (squKD), from the Vienna Drosophila RNAi Center. The hairpin line for Armitage knockdown has one predicted off-target site [plexin B (CG17245), a gene that functions in axon guidance and is unlikely to contribute here]. The hairpin lines for Squash, Piwi and Aub have no other predicted targets. The GAL4 driver line used is *P{w*$^{+mC}$*=UAS-Dcr-2.D}1, w*^{1118}*; P{w*$^{+mC}$*=GAL4-nos.NGT}40* from Bloomington Stock Center. The *piwi*^{NT}*, cn*^{1}*, bw*^{1} */CyO* line was a gift from Alexei Aravin (California Institute of Technology,

Pasadena CA). The y^1w^{67C23} line is used as a wild type control. To drive female germ-line knockdown of a gene product, male flies from the driver line were crossed with female virgins from the respective hairpin target lines (or *yw* as control). Ovaries were dissected from 3- to 5-day-old females provided with fresh yeast overnight. Because homozygous females are infrequently recovered from the $piwi^{NT}$ line, the collection window was extended up to a week.

Immunofluorescent imaging

Ovaries were dissected in EBR (an iso-osmotic buffer) and fixed in 6% formaldehyde saturated with heptane (Cooley et al., 1992). P4D2 antibody (Saito et al., 2006) (1:2 in PBT) was used for Piwi immunostaining. Alexa Fluor-conjugated secondary antibodies used are from Invitrogen. Images were collected on a Nikon A1 confocol microscope.

Quantitative PCR profiling of transposon expression

Transposon expression profiles were generated as previously described (Wang and Elgin, 2011). Briefly, total ovarian RNA is isolated using TRIzol and treated with DNase I (Fermantas) before use for cDNA generation. cDNA is made using SuperScript III with random hexamers (Invitrogen). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a ABI 7500 Real-Time

PCR system. Primers used are listed in Supplemental Table 1. Results were analyzed by using the ddCT method (Livak and Schmittgen, 2001).

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Figure legends

Figure 1. Knockdown of Squash or Armitage in the female germline has different impacts on transposon expression. (a, b) Quantitative RT-PCR analysis of gene/ transposon expression levels in germline Squash/ Armitage knockdown ovaries. Expression levels are given relative to the RPL32 locus. (Bars represent the

mean ± SEM.) (a) demonstrates effective knockdown of the target mRNA. (b) gives mean levels of transposon expression determined from three biological replicas for each sample. (c) Piwi immunofluorescent staining of ovarioles. In the Armitage knockdown germline, Piwi is predominantly localized in the cytoplasm, in contrast to wild type where it is concentrated in the nucleus. (Scale bars: 50 μm.)

Figure 2. Nuclear localization of Piwi is critical for silencing most transposons. Quantitative RT-PCR analysis of transposon expression levels in *piwi*^{NT} ovaries. Expression levels are given relative to the RPL32 locus. (Bars represent the mean ± SEM.)

Figure 3. Piwi, Aub double knockdown reveals complex epistatic interactions. (a, b) Quantitative RT-PCR analysis of gene/ transposon expression levels comparing ovaries subject to germline Piwi knockdown, Aub knockdown or Piwi, Aub double knockdown. Expression levels are given relative to the RPL32 locus. (Bars represent the mean ± SEM.) (a) demonstrates effective and independent knockdown of the target mRNAs. (b) gives mean levels of transposon expression determined from three biological replicas for each knockdown sample.

Figure 4. Model depicting genetic interactions between secondary piRNA-dependent and -independent pathways. The latter pathway is most likely

dependent on primary piRNAs. *HeT-A* and *Invader1* are the representative examples of transposons that are respectively silenced only by either secondary piRNA-dependent or -independent pathways. Redundant silencing by Aub- and by Piwi (in the secondary piRNA-independent pathway) between the two pathways could result in the synergistic effect observed for *Jockey* in the double knockdown ovaries.

Table 1 Comparison of the transposon expression profiles resulting from mutations in PIWI family proteins

element	PiwiKD [a]	AubKD [a]	*ago3* [b]
1731	+	+	+
Blood	+++	+++	++++
Burdock	++++	++++	++++
HeT-A	++++	++++	+++
Invader1	++	-	-
Invader4	+++	+	+
Jockey	+	-	+
Max	++	-	-

* Normalized expression level relative to an appropriate wildtype control is summarized for each transposon. - indicates 0 ~ 2-fold change, + indicates 2~5-fold increase, ++ indicates 5~10-fold increase, +++ indicates 10~50-fold increase, ++++ indicates a more than 50-fold increase.

a. Data summarized from Wang and Elgin, 2011
b. Data summarized from Lee et al, 2009

Table 2 Comparison of the transposon expression profiles resulting from germline knockdown of piRNA pathway components

element	PiwiKD [a]	ArmiKD	AubKD [a]	SquKD
1731	+	+	+	++
Blood	+++	+++	+++	++++
Burdock	++++	++++	++++	++++
HeT-A	++++	++++	++++	++++
Invader1	++	-	-	-
Invader4	+++	+++	+	++
Jockey	+	+	-	-
Max	++	+	-	-

* Normalized expression level relative to an appropriate wildtype control is summarized for each transposon. - indicates 0 ~ 2-fold change, + indicates 2~5-fold increase, ++ indicates 5~10-fold increase, +++ indicates 10~50-fold increase, ++++ indicates a more than 50-fold increase.

a. Data summarized from Wang and Elgin, 2011

Fig 1a

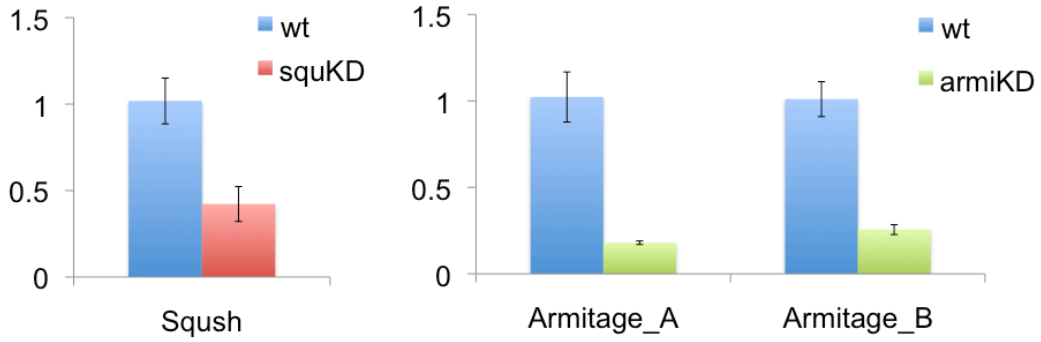

1.5
1
0.5
0
wt
squKD
Sqush
1.5
1
0.5
0
wt
armiKD
Armitage_A
Armitage_B

Fig 1b

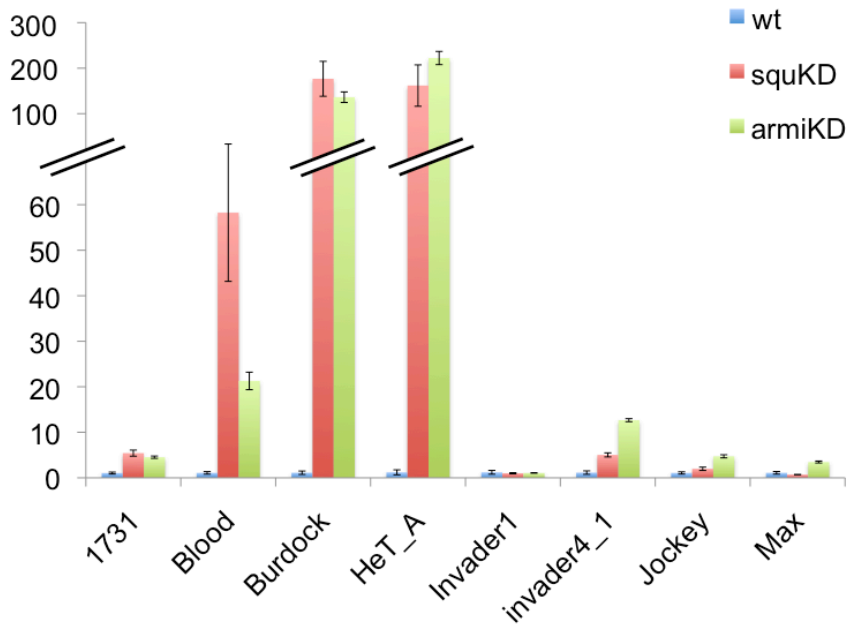

300
200
100
60
50
40
30
20
10
0
wt
squKD
armiKD
1731
Blood
Burdock
HeT_A
Invader1
invader4_1
Jockey
Max

Fig 1c

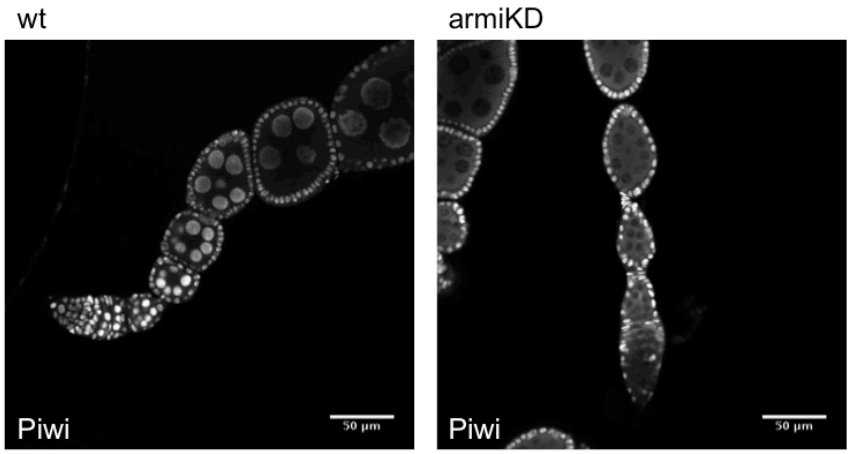

wt
armiKD
Piwi
50 µm
Piwi
50 µm

Fig 2

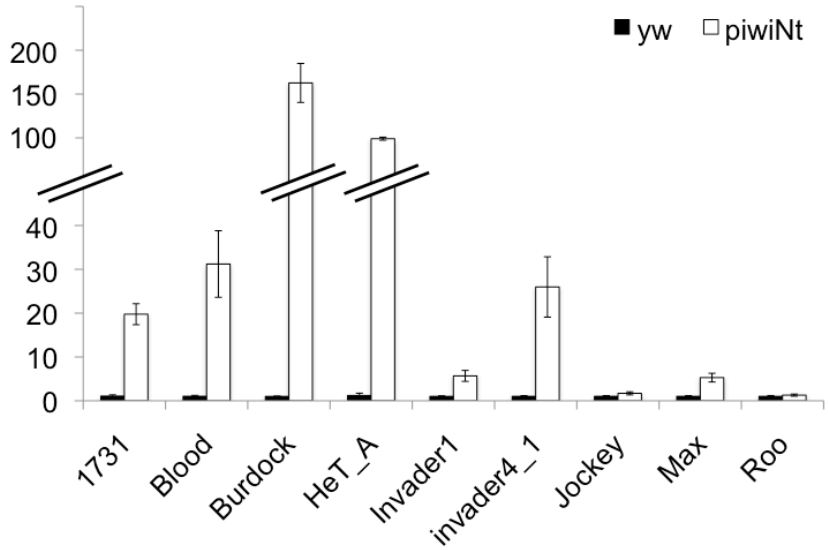

yw
piwiNt
200
150
100
40
30
20
10
0
1731
Blood
Burdock
HeT_A
Invader1
invader4_1
Jockey
Max
Roo

Fig 3a

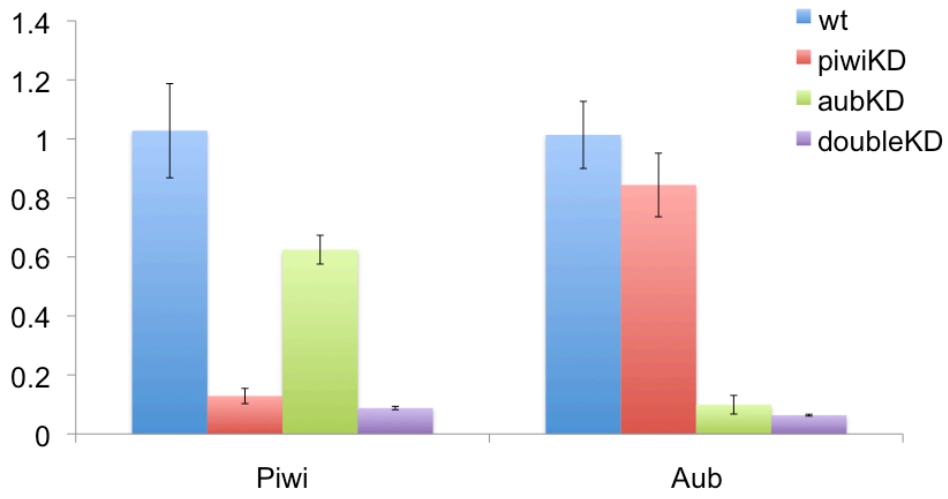
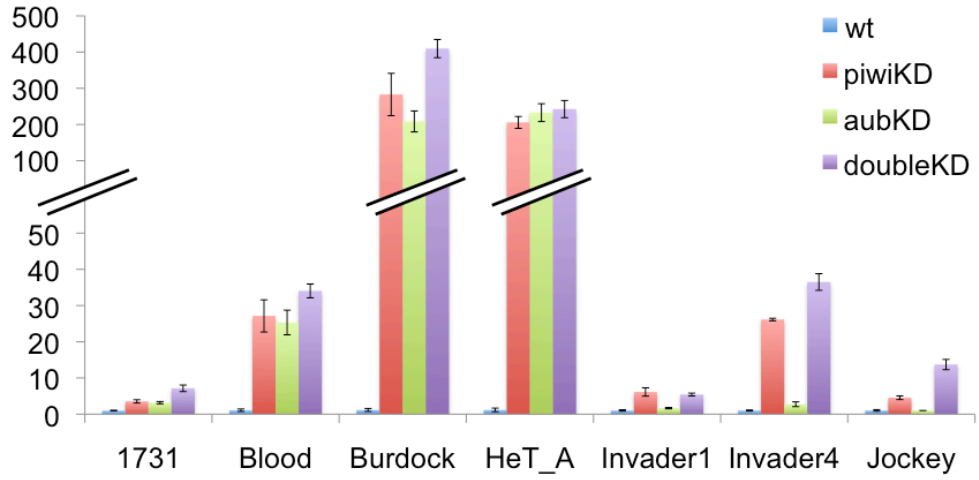

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Piwi
Aub
wt
piwiKD
aubKD
doubleKD

Fig 3b


500
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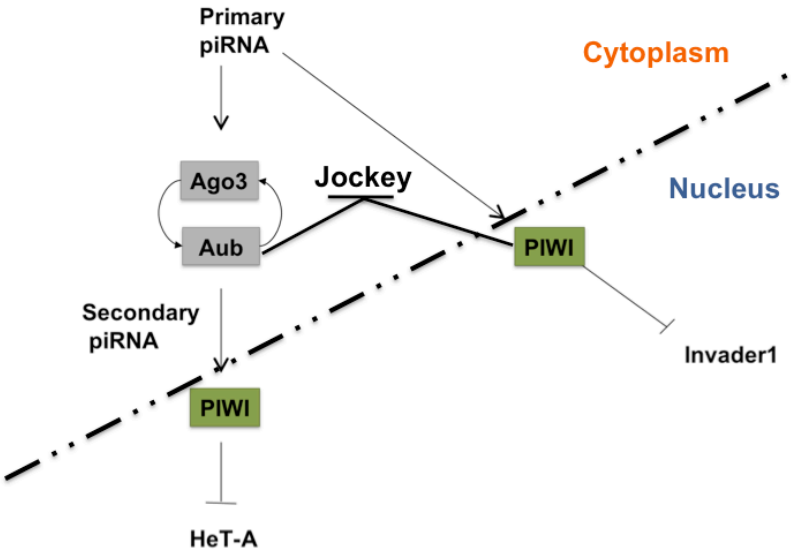
Blood
Burdock
HeT_A
Invader1
Invader4
Jockey
wt
piwiKD
aubKD
doubleKD

Fig 4


Primary
piRNA
Cytoplasm
Ago3
Jockey
Nucleus
Aub
PIWI
Secondary
piRNA
Invader1
PIWI
HeT-A

Chapter 6

Conclusions and future perspectives

Silencing by heterochromatin formation appears to be tightly regulated by both the cis-regulatory elements at the locus of interest and the trans-acting elements, which encode proteins and RNAs critical for the process. The observations that I have made using inbred PEV reporter lines (Chapter 2) strongly support the view that many aspects of this process are genetically determined. A precise mechanism to designate regions of the genome for heterochromatin formation is therefore encoded in the genome itself, albeit with some stochastic features. Building on this observation, I further pursued the function of a small-RNA binding argonaute protein, Piwi, demonstrating a role in heterochromatin formation at a subset of transposable elements. The selectivity observed is most readily explained by using the sequence information encoded in the small RNAs to recognize the corresponding genomic locus. While results from my research have indicated a clear role for Piwi (and by implication, Piwi-interacting RNA) in targeting transposons for heterochromatin silencing, much remains to be elucidated to describe this pathway. In particular, details regarding both the targeting (silencing) mechanism utilizing piRNA and the biogenesis of piRNA remain unclear. Whether piRNA can function outside of the reproductive system, and whether it works in collaboration or is redundant with other types of targeting mechanisms (as seen in the fission yeast *S. pombe*) is also unclear. Identification of additional factors involved in the process of heterochromatin targeting/ formation will help fill in these gaps. The experimental systems developed here can serve as an excellent foundation for further pursuit of a better understanding of heterochromatin targeting and piRNA biology.

PEV is a quantitative trait

Making the fly line 39C12 inbred eliminates much of the variation in a PEV phenotype observed using the *white* gene as a reporter. This is true in terms of both the level and pattern of silencing in the adult fly eye. Similar results were accomplished by using balancers to “clean up” the genetic background. The detailed characteristics of the PEV phenotype (and the heterochromatin silencing it represents) are therefore determined largely by genetics (assuming that environmental factors such as temperature, humidity, population density, etc., are held constant) and the degree of silencing is therefore a quantitative trait. Although a genetic modifier of PEV is not a new concept, our results clarify the relationship between the commonly seen high variability of a PEV phenotype and the genetic/epigenetic influences. A quantitative trait locus (QTL) mapping study is therefore applicable to PEV using its associated phenotypes.

Much of our understanding of heterochromatin structure and function derives from analysis of mutant alleles recovered using screens for PEV modifiers (Wustmann et al., 1989; Hayashi et al., 1990; Girton and Johansen, 2008). While a handful of Su(var) mutations have been carefully studied, a large collection of mutant lines recovered from these modifier screens remain to be characterized. In most cases the critical mutations have only been mapped to the

chromosomes, primarily because of the extensive resources required to pinpoint the causative mutation. Increasing the throughput and confining the search space for the identification process could remedy these issues. To increase the throughput, pooled genomic DNA samples (from these mutant lines) can be analyzed using a multiplexed next generation sequencing strategy. To confine the search space, a set of high resolution QTL (Quantitative Trait Loci) mapped as modifiers of PEV of the same reporter (used in the EMS screens) could be used to focus the sequencing analysis on a set of loci (with defined intervals) of interest. Although this approach will exclude the possibilities of identifying mutations from the screen that do not overlap with the regions defined by QTL mapping, the investment/recovery ratio will be much lower than sequencing hundreds of fly genomes, not to mention the comparison to traditional methods for mapping the causative mutations.

Given the relative ease of rearing large numbers of fruit flies and visually inspecting PEV phenotypes, I propose that a bulk segregant analysis type of QTL mapping would be most suitable for this purpose. The A and D lines established here (Chapter 2) could be used; as they are derived from a single population, there will be relatively few polymorphisms between the A and D genomes. Low levels of variation between the genomes of mapping populations would be beneficial in identifying the causative polymorphism; however, at the same time fewer polymorphisms could also mean that we could miss important factors in the mapping process. Additional inbred lines could also be used for mapping; as an

alternative. w^{1118} appears to be suitable for this purpose since it is already inbred and is *white* minus. The pattern of PEV should be treated as a separate trait for mapping, distinct from the level of expression. Visual inspection of the fly's eye is again the best approach to accomplish this. Many inferences have been made based on how the patterns of PEV might reflect the timing and stability of epigenetic inheritance of the chromatin state (Tartof et al., 1984). However, lack of careful documentation and tangible approaches to analyze the details of the process have left most of these inferences as speculations. Identification of genetic factors determining both the patterns of PEV and the stability of those patterns will provide a means to understand this process. Using a widely-expressed β-galactosidase marker, Lu et al. have been able to demonstrate that the variegation observed occurs as a consequence of a relaxation of silencing during pupation (Lu et al., 1998). It will be interesting to further characterize genes identified to impact stability of PEV patterns in this context.

As is the case for EMS-induced mutations (and many other types of screens), when identifying the causative polymorphisms (mutations), population genetics approaches are exon centric. This is largely the consequence of a lack of a well-defined 'genetic code' for most of the genome. As demonstrated here (and elsewhere), small RNAs and other non-coding elements play critical roles in the targeting and silencing of heterochromatin. Polymorphisms in the "non-coding" regions of the genome therefore are likely to play a role in the phenotypic outcome scored using PEV. The genetic code helps to identify causative

mutations by excluding synonymous mutations from consideration and focusing attention on amino acid substitutions, particularly those that alter the character of the amino acid. As a consequence of a more confined search space, statistical power increases. Similarly, for the non-coding regions, correlations with genome annotations such as conservation scores from closely related species, and certain chromatin signatures (such as the presence of DNase hypersensitive sites, indicating open chromatin) could be useful in facilitating the identification of causative mutations.

Reporter insertions in different regions of heterochromatin appear to show distinct response profiles to well-characterized PEV modifiers (Cryderman et al., 1999; Haynes et al., 2007). The analysis presented here for the Ys telomeric heterochromatin provides an excellent example, illustrating the diversity in subtypes of heterochromatin (Chapter 3). While considerable effort has been made to survey different regions of the genome, these analyses are usually performed with a limited numbers of modifiers, and interpretation of each data point is confounded by potential background modifiers (Cryderman et al., 1999; Haynes et al., 2007). Using the unique genetic tools available in the fruit fly, substituting reporter chromosomes into an inbred line without perturbing the genetic background is possible (see examples in chapter 2). It is therefore possible to map QTL for PEV from different reporter insertions using the same mapping population. Results form this type of analysis could be used to

conclusively categorize different genomic regions as to subtypes of heterochromatin.

A population genetics approach to studying PEV also circumvents the issue of unrecognized background modifiers present in the analysis, and has the potential to identify genetic interactions between loci with higher confidence. Interactions between PEV modifiers represents an area that is underdeveloped in the field. This type of research could be useful in delineating the regulatory network that drives heterochromatin formation and in identifying novel pathways involved in the process.

Using small RNA to target heterochromatin formation

Germline Piwi depletion leads to transposon over-expression and a corresponding loss of HP1a enrichment at a subset of transposable elements (Chapter 4). Accordingly, depletion of HP1a in the germline also shows similar over-expression from the same set of transposons. These observations are indicative of a small RNA targeting mechanism for heterochromatin formation. A mechanism involving Piwi recruitment of HP1a is especially compelling when considering the direct interaction between Piwi and HP1a (Brower-Toland et al., 2007; Mendez et al., 2011). It is however, unsettling to discover that a valine to alanine substitution in the Piwi N-terminus that disrupts the direct interaction between the two proteins in a yeast two-hybrid system has no observable

consequence *in vivo* (Chapter 4). To encompass these two seemingly contradictory observations, a bridging protein of unknown identity is proposed to function to stabilize the Piwi-HP1a interaction, keeping a small RNA targeting complex together. The role of this protein is envisioned to be similar to the Tas3 protein in the *S. pombe* RITS complex (Verdel et al., 2004).

Conceptually, a pull-down experiment appears to be the obvious approach to identify the bridging protein. In reality, biochemical approaches to studying chromatin-associated proteins have not always been straightforward. Multiple attempts to identify an HP1a complex have been without success (Elgin lab and others). Similarly, attempts to identify a Piwi complex using ovary lysate have also failed to recover stable interacting proteins (Brent Brower-Toland, personal communication and others). Using ovarian somatic cell lines, two independent groups have reported a Piwi complex involving piRNA pathway components Armitage, Yb and Squash (Haase et al., 2010; Saito et al., 2010). Despite the predominant nuclear localization pattern of Piwi, all of these proteins recovered with Piwi by immunoprecipitation are cytoplasmically localized, with a particular enrichment in an organelle called the ‘Yb body’ (Saito et al., 2010). Neither group recovered HP1a in this complex.

Although identification of the bridging protein would close one important gap in the Piwi targeting model, the task could be daunting without a better strategy for narrowing down the candidate list. Alternative strategies focusing

directly on the ability of Piwi to target induction of heterochromatin formation are likely to be more productive. One on-going project in the Elgin lab is looking at bypassing the small RNA in the targeting process by tethering Piwi to a specific site in the genome [using the *lacO* system (Robinett et al., 1996)] to induce ectopic heterochromatin formation. While the results will most likely be context dependent, a proof of principle showing induction of ectopic heterochromatin would confirm a function for Piwi in such targeting, and provide a platform to further dissect the domains of Piwi to determine what functions are required in the downstream recruitment process. For example, in *S. pombe*, slicing of the nascent transcript is a critical step for heterochromatin formation (Irvine et al., 2006). It will be interesting to see if the slicer domain of Piwi might play a similar role in flies.

Another disconnect between the germline study of Piwi presented here and the current model of heterochromatin formation is found in the developmental timing. The prevalent model describes the initiation of heterochromatin formation during the early stages of embryogenesis (nuclear cycle 11-14) (Vlassova et al., 1991). Large amounts of piRNA, of PIWI, and of numerous chromosomal proteins are loaded into the oocyte, and so are present in the early embryo prior to zygotic gene expression; this is reported to be critical for transposon suppression and prevention of hybrid dysgenesis (Brennecke et al., 2008). Whether Piwi and piRNA play a role in heterochromatin formation in the early zygote remains to be demonstrated. The maternal loading of Piwi

protein into the egg makes a conditional depletion in the early zygote difficult with current technology. A depletion of maternal Piwi results in dead embryos. Nonetheless, RNAi-based knockdown of Piwi in the early zygote does show some long-lasting impacts on a PEV phenotype in adult tissues (Tingting Gu and SCRE, personal communication). Perhaps a more direct approach here would be to deplete specific piRNAs in the early zygote by developing strategies similar to the miRNA 'sponge' (Ebert et al., 2007). While redundancy and influences from neighboring regions are likely issues, and could mask the impact at a specific test site, by carefully designing the sequences of the piRNA sponge, this approach has the potential to allow analysis of impacts on chromatin structure with minimal pleotropic consequences.

Homozygous *piwi* mutant flies are viable (but infertile). In contrast, a lack of HP1a (achieved by a heteroallelic cross) leads to a lethal arrest at the early pupal stage. How flies survive without Piwi is an interesting question, especially when considering Piwi's function in targeting heterochromatin. Heterochromatin is particularly important in mitosis, as it is required for marking the centromere. Homozygous *piwi* flies are recovered from a heteroallelic cross; as a large amount of Piwi protein is loaded into the oocyte and present in the early zygote, the *piwi* mutants do have some Piwi during the early stages of embryogenesis. Whether the maternal loading of Piwi is sufficient to sustain the fly, which would imply a stable mitotic inheritance of heterochromatin after the initial pattern is set, or alternatively, there are redundant mechanisms that substitute for Piwi remains

to be clarified. Recently, endo-siRNA has been implicated in targeting heterochromatin formation (Fagegaltier et al., 2009). It will be interesting to see if there are synthetic phenotypes when combining mutations from the two distinct pathways.

Better understanding of piRNA pathways

By closely examining the transposon responses to mutations in PIWI famlily proteins, we conclude that in addition to a function downstream of secondary piRNA (Chapter 4), germline Piwi can also have a role in transposon silencing independent of secondary piRNA (Chapter 5). Considering the current literature (Malone et al., 2009; Saito et al., 2009), we propose that as is the case for Piwi in ovarian soma, germline Piwi could function in transposon silencing by directly utilizing primary piRNAs. In an attempt to identify genes that function specifically in this primary piRNA pathway, I found that knockdown of Armitage or Squash in the female germline recapitulates the dichotomy between germline Piwi or Aub knockdown, as seen by creating a ‘profile’ of the responses of several different TEs (Table 2, Chapter5). While our results on Armitage support previously published findings that indicate a role for Armitage in piRNA loading, little functional inference can be made concerning Squash. Key questions remaining include whether it’s putative RNaseHII domain is required for transposon silencing. Further investigation of Squash has the potential to unveil

the downstream process of piRNA silencing (the effector phase) (Haase et al., 2010).

Unexpectedly, I also noticed one transposon, *Jockey*, that appears to be regulated by Piwi in the cytoplasm, as cytoplasmically localized Piwi is sufficient for *Jockey* silencing (Chapter 5). Note that expression of *Jockey* is not sensitive to the germline depletion of HP1a (Chapter 4). Although *Jockey* is the only case among those studied here that shows such properties, singular cases are often the window to a scientific breakthrough. Further investigations of *Jockey* silencing promises discoveries of additional modes of Piwi-dependent silencing. One obvious question that stems from this observation is whether cytoplasmic Piwi utilizes its slicer domain in this (or other modes) of transposon silencing. My attempt to generate informative Piwi mutant transgenes, specifically to investigate the slicer function and the RNA binding function, did not result in constructs with consistent stable protein expression (Appendix). However, an ongoing collaboration between the Elgin lab and the Haifan Lin lab at Yale University is working towards resolving this issue.

Our knowledge regarding how piRNA is made remains limited. Sequencing of piRNA libraries has led to the identification of many sites in the genome that serve as the source of piRNAs (piRNA loci or piRNA clusters); these primary transcripts are evidently processed in more than one way, including the secondary generation of piRNAs through the ‘ping-pong’

mechanism (see Chapter 1). At this point, only the generation of the 5'-end of secondary piRNA is well-characterized (Brennecke et al., 2007; Gunawardane et al., 2007). The maturation of piRNA 3' -end is proposed to be a result of a competition between trimming by a yet to be identified exonuclease followed by 2' O-methylation by Hen1, which stops the trimming process (Kawaoka et al., 2011). The biogenesis pathway for primary piRNA is largely unknown; it has been proposed that primary piRNAs are derived from single-stranded long transcripts made by PolII from the piRNA clusters in a process involving Zucchini (Saito et al., 2009). A better understanding of piRNA biogenesis will obviously benefit our understanding of the downstream silencing processes. A detailed mechanistic understanding of piRNA biogenesis will require an understanding of the underlying enzymology, as has been developed for DNA replication and transcription. *In vitro* piRNA production using purified fractions from ovarian somatic cell lines has the potential to reveal detailed mechanisms of piRNA production, and such a system needs to be developed. Given the new technologies available for proteomics, this would greatly facilitate identification of key factors involved in the process. In fact, a system for these types of studies has already been developed using lysates from a silkworm ovarian cell line, BmN4 (Kawaoka et al., 2011). Given the promising initial observations on piRNA 3' end formation, exciting new discoveries describing a detailed mechanism of piRNA biogenesis seem likely to emerge. Similarly, with improved understanding of the piRNA biogenesis process, this type of analysis could also be applied to investigate the mechanism of piRNA transposon silencing.

Although much of the published literature on piRNA focuses on its role on transposon silencing in the reproductive system, another interesting observation on piRNA pathway components is their enriched expression in fat body and CNS. The functional meaning of this expression remains to be explored. Small RNA matching the characteristic features of piRNA has been found in fly heads (Ghildiyal and Zamore, 2009). Recently, a role for “piRNAs” in the *Aplysia* CNS has been reported (Rajasethupathy et al., 2012). While much remains to be confirmed, a piRNA function outside of the reproductive system and in addition to that transposon silencing is likely. piRNAs derived from sequences unrelated to transposons have been reported (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). In particular, some piRNA has been found enriched in the 3’ UTR of annotated genes with known functions (Robine et al., 2009; Saito et al., 2009). Further investigation of these piRNAs using sequence-specific piRNA depletion strategies, as discussed above, will likely unveil new functions of piRNA in addition to transposon control.

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Appendix

Construction of transgenic fly lines for functional analysis of Piwi

Introduction

Piwi is a nuclear-localized argonaute protein that functions to silence transposons in the reproductive system through its interaction with a group of small RNAs, piRNA (Saito et al., 2006). Similar to other argonaute proteins, Piwi is predicted to have four functional domains that cooperate to play critical roles in Piwi function. Initial functional tests of these domains has already been accomplished in a cell culture system (Saito et al., 2009, 2010). It was shown that the piRNA binding function of PAZ domain is required for transposon silencing, while the slicer function of the PIWI domain is dispensable (Saito et al., 2009, 2010). In addition, the N-terminal 13 residues of Piwi are required for Piwi nuclear localization, and are required for its functions in transposon silencing (Saito et al., 2009). While these results delineate an intriguing picture of how Piwi might function in transposon silencing in the nucleus through a mechanism independent of putative slicer activity, it remains unclear how these observations would be recapitulated in the intact organism.

In the reproductive system, Piwi functions in transposon control in both the germline and the ovarian soma (Malone et al., 2009; Saito et al., 2009). In the ovarian soma, Piwi likely functions in a fashion similar to what is described by Saito and colleagues in the cell culture system, while in the germline the system is more complicated. In addition to Piwi, two additional PIWI family proteins, Aub and AGO3, function in secondary piRNA production, which is specific to germline

(Brennecke et al., 2007; Gunawardane et al., 2007). By depleting Piwi specifically in the germline, I found that Piwi could function downstream of secondary piRNA production to achieve transposon silencing through an HP1a-dependent mechanism (Chapter 4). In addition, I have also shown that Piwi could also function in a process independent of secondary piRNA in the germline (Chapter 5). How these two different Piwi functions are distinguished in the germline remains unclear. Distinctions of subcellular localization represent one promising possibility. Our effort in answering these questions can significantly benefit from constructing a set of transgenic fly lines expressing informative mutant Piwi.

Results

Given our interest in dissecting the functional distinctions between the two germline pathways utilizing Piwi, the six transgenic constructs generated and characterized by Saito and colleagues appear to be a great starting point (Saito et al., 2009, 2010). The Saito constructs are modified from a cDNA clone (Rubin et al., 2000) to incorporate a MYC tag at the N-terminus just after the first residue, and each of the designated mutations. The ΔN mutant has the N-terminal 70 residues truncated, while the ΔN13 only removes 13 residues. Point mutations DDAA and ΔPAZ respectively abolish the putative slicer function of the PIWI domain and the piRNA binding function of the PAZ domain. The wildtype construct has been shown to express MYC-tagged protein that co-

immunoprecipitates with piRNAs and is functional in rescuing knockdown phenotypes in the ovarian somatic cell line (Saito et al., 2009). To enable a tissue-specific expression of these transgenes in flies, I PCR-amplified each MYC-Piwi fragment from the Saito constructs and cloned them respectively into a VALIUM22 expression vector (Ni et al., 2011). In addition to the *UAS-P-transposase-promoter* element, which allows tissue-specific expression when combined with different GAL4 drivers, VALIUM22 also allows site-specific integration using the *phi-C31* integrase to ensure consistent expression levels. In addition, the *vermilion* marker used for screening transgenic flies is such that it allows downstream application of most PEV assays.

Six transgenic constructs were made and each integrated into one specific site on the X chromosome. Integration at the same site can ensure consistency of expression; using the X chromosome allows genetic manipulations for a germline-specific rescue experiment for analyzing the functional relevance of each mutated domain (similar to what was done for the $piwi^{V30A}$ transgene in Chapter 4). Sequencing of PCR fragments amplified from the genomic DNA prepared from each transgenic fly line showed only the expected mutations (Fig 1). However, unfortunately, preliminary testing on expression of these transgenes using western analysis shows a lack of protein product (Figure 2a). In fact, driving the expression with the germline specific driver NGT40 resulted in mostly degradation products when visualized with an anti-cMYC antibody (Figure 2a). Only trace amounts of protein product of the expected size are seen for both

wildtype and N-terminal truncation lines (Figure 2a). In contrast, a MYC-tagged Piwi transgeneic line previously described by the Haifan Lin lab (Cox et al., 2000; Megosh et al., 2006) shows a single product of the expected size. Similar to the results from using a MYC antibody, P4D2 monoclonal antibody raised against the Piwi N-terminus shows the degraded protein products in addition to the endogenous Piwi (Figure 2b). Given the smaller size products recognized by both the MYC-tag and the P4D2 antibodies, we infer that the protein products expressed from these transgenic lines are not stable.

Consistent with the observations made from western analysis, rescue experiments using the wildtype transgene made here show variable partial rescue. Instead of a highly penetrant rescue from the arrest phenotype during embryogenesis typical of germline *piwi* mutants (Cox et al., 1998), adding this wildtype transgene in combination with an *NGT40* driver results in a low rate of rescue with some hatched larvae. Use of a stronger driver *NGT40; NGTA* results in an increased rate of rescue, and a small fraction of the progeny develop to adulthood. Although increased driver strength does result in some progeny surviving to eclosion, the low rate of rescue (less than one progeny eclosed per cross) and variable arresting stages observed suggests high variation between individuals, which made a quantitative assessment of impact unachievable.

Discussion

While it remains unclear how the protein products of these transgenes are subjected to degradation, it is clear that these transgenic fly lines are not suitable for the analysis they were intended for. As an alternative, one could construct the transgenes based on the transgenic constructs from the Haifan Lin lab (Cox et al., 2000). Recently, we are made aware of the fact that the Lin lab has already constructed fly lines expressing Piwi with either the PAZ domain or the PIWI domain mutation. Using these fly lines together with the N-terminal truncation mutant recently published (Klenov et al., 2011), researchers can start to reveal the function of the individual domains and how they impart Piwi function *in vivo*.

Materials and Methods

Construction of transgenic fly lines

MYC-Piwi fragments were PCR amplified from the Saito constructs (Saito et al., 2009, 2010) using Klentaq LA (DNA Polymerase Technology) with the following primer pair: forward, AT TCTAGACATGGG AGA GCA GAA ACT GAT C; reverse, AT GCGGCCGC TTATAGATAATAAAACTTCTTTTCGAG. The PCR fragments were then digested and cloned into the VALIUM22 plasmid (Ni et al., 2011) between the *Xba*I and *Not*I sites. The plasmids were amplified in *E. coli* and Midi (Qiagen) prepped for injection into a fly line BL34769 (y^1,P{$y^{+t7.7}$=CaryIP}su(Hw)attP8,v^1). The injections were done by Rainbow Transgenic Flies and the transformants were made into a stock and kept using

standard balancers. For the expression test, each transgenic line (female) was crossed to driver lines BL4442 (y^1 w*; P{w^{+mC}=GAL4-nos.NGT}40) and BL32564 (y^1 w*; P{w^{+mC}=GAL4-nos.NGT}40; P{GAL4-nos.NGT}A).

Western analysis

Five pairs of ovaries dissected from 4-day-old females are hand homogenized in RIPA150 to prepare ovarian lysate for western analysis. A 4-20% polyacrylamide gradient gel (Bio- Rad) is used for separation; the proteins were then wet-transferred to a nylon membrane. P4D2 Piwi antibody (1:100) and c-MYC antibody ab9106 (Abcam) (1:5000) are used in 5% milk/TBST to probe for transgenic Piwi proteins. HRP (horseradish peroxidase) conjugated secondary antibodies (KPL) and substrates (Millipore) were used to visualize the results.

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Figure Legends

Figure 1. A multiple sequence alignment showing the sequencing results confirming that the correct constructs are present in the transgenic fly lines. In every case tested we see only the expected mutations. The color-coded bars on top of the sequence alignment are used to highlight the regions of mutations (red, no mutations; green, two constructs contain mutations; blue, three constructs contain mutations). a. Alignment of the N-terminal region of the Piwi transgenes including the Myc-tag and some upstream sequences to demonstrate the presence of the intended truncations. b. Sequence alignment showing one of the point mutations in the PIWI domain disrupting the putative slicer activity.

Figure 2. Results from western analyses indicate that the protein products expressed from the Piwi transgenes are degraded. a. Probing with MYC antibody show only a trace amount of signal for WT and ΔN Piwi at the expected size (arrow heads) and a smaller size product (from every transgene) indicative of protein degradation. Note the strong band of expected size in the first lane from a fly line expressing MYC-tagged Piwi previously published (used as a reference). b. Probing with Piwi antibody shows a predominant signal from the endogenous Piwi (arrow head) and the ladder-like signals at the lower area of the blot, likely

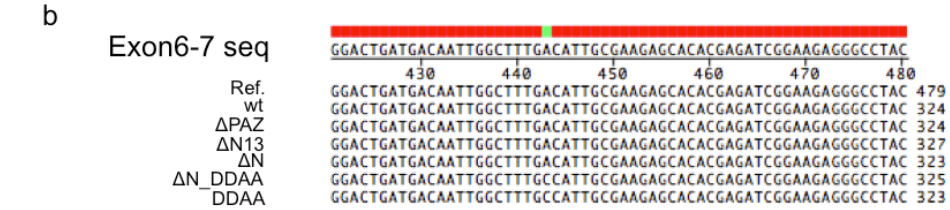
coming from degraded Piwi proteins. Note that the lysate from the reference MYC-Piwi line does not show the signal indicative of degradation products.

Figure 1

a


N-terminal seq
CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTTGCTGATGATCAGGG
150 160 170 180 190 200 210
Ref. CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTTGCTGATGATCAGGG 206
wt CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTTGCTGATGATCAGGG 208
ΔPAZ CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTTGCTGATGATCAGGG 209
ΔN13 CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTT---------- 197
ΔN CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTT---------- 195
ΔN_DDAA CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTT---------- 191
DDAA CTGTTCTAGACATGGGAGAGCAGAAACTGATCTCTGAAGAAGACCTGAACAAGCTTGCTGATGATCAGGG 208
ACGTGGACGCAGGCGTCCACTTAACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG
220 230 240 250 260 270 280
Ref. ACGTGGACGCAGGCGTCCACTTAACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG 276
wt ACGTGGACGCAGGCGTCCACTTAACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG 278
ΔPAZ ACGTGGACGCAGGCGTCCACTTAACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG 279
ΔN13 ----------AACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG 244
ΔN ---------- 195
ΔN_DDAA ---------- 191
DDAA ACGTGGACGCAGGCGTCCACTTAACGAAGATGATTCCTCTACTTCCCGAGGTAGTGGTGATGGGCCGCGG 278

b


Exon6-7 seq
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430 440 450 460 470 480
Ref. GGACTGATGACAATTGGCTTTGACATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 479
wt GGACTGATGACAATTGGCTTTGACATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 324
ΔPAZ GGACTGATGACAATTGGCTTTGACATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 324
ΔN13 GGACTGATGACAATTGGCTTTGACATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 327
ΔN GGACTGATGACAATTGGCTTTGACATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 323
ΔN_DDAA GGACTGATGACAATTGGCTTTGCCATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 325
DDAA GGACTGATGACAATTGGCTTTGCCATTGCGAAGAGCACACGAGATCGGAAGAGGGCCTAC 323

Figure 2a

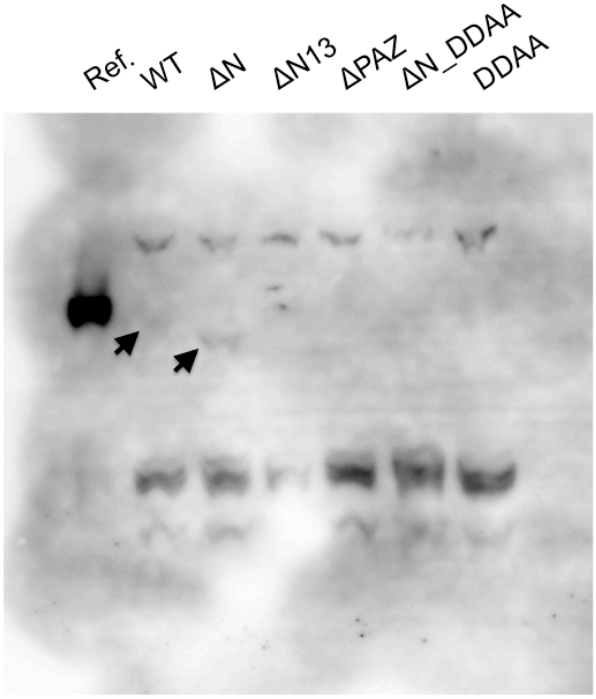

Figure 2b